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A METHOD FOR MEASURING THYROID HORMONE SECRETION IN THE RAT WITH ITS APPLICATION TO THE BIOASSAY OF THYROID EXTRACTS

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INTRODUCTION

THE work reported in this paper deals with a method of measuring the secretion or release of hormone by the thyroid gland of the rat. The procedure depends on the administration of a tracer dose of radioactive iodine to the experimental animals and after organic binding of the iodine in the thyroid measuring by means of daily in vivo counts, the decline in radioactivity of the thyroid gland, with appropriate corrections for isotopic decay. This rate of decline is taken to be an index of hormone secretion by the gland.

Thyroid secretion has been previously measured by methods based on the amount of thyroid hormone or thyroxine required to prevent the development of thiouracil goitres (Dempsey and Astwood, 1943; Reineke *et al.*, 1945; Hurst and Turner, 1947). Such methods though simple to carry out require some time; the method presented below matches these procedures as regards ease of performance and in addition offers the further advantage of speed.

METHOD

Male albino rats from a highly inbred strain five to six months of age weighing 170–250 gms. were used. These animals had been maintained since weaning on a diet which though low in iodine (500 μg.I/Kgm.) was only slightly goitrogenic and was capable of supporting normal growth and reproduction. After withholding food 15 hours the animals were given an intraperitoneal injection of 20–25 microcuries of carrier-free I^{131} as NaI. Twenty-four hours later, at which time the majority of the radioiodine in the thyroid is in organic combination (Taurog and Chaikoff, 1947) the radioactivity of the thyroid gland was measured in vivo by firm application of a Geiger-Mueller tube to the neck region, the rats being first anesthetized with nembutal and placed supine on

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a board. The tube had a mica window 2.5 cm. in diameter covered with an aluminum shield 2 mm. thick. The tube was fixed in contact with the animal's neck until at least 1200 counts were recorded.

Following counting the animals were divided into control and experimental groups, four animals per group, so as to include approximately equal numbers of animals with high and low counting rates in each group. They were then returned to their cages and placed on one or other of the following regimes; propyl thiouracil 0.2% of the diet, KSCN 0.4% of the diet, KI 0.5% of the diet, l-thyroxine 15 μg. injected subcutaneously daily, thyrotrophic hormone (Armour) 9 mgm. subcutaneously daily. The effect on the radioactivity of the gland was determined by counting as described above at 24 hour intervals over a five to seven day period.

After correction for isotopic decay the daily thyroid counts of each animal were expressed as a per cent of the initial count taken 24 hours after the tracer dose.

TABLE 1. THE RELEASE OF RADIOIODINE FROM THE THYROID GLAND OF THE RAT OVER A FIVE DAY PERIOD

Rat	% dose taken up at 24 hrs.	% of initial activity (24 hr. uptake) on day					
		0	1	2	3	4	5
1	22.4	100	83	54	41	29	23
2	24.0	100	65	38	31	20	15
3	20.5	100	73	50	34	25	16
4	22.6	100	67	42	27	19	14
Average		100	72	46	33	23	17

RESULTS

In Table 1 are shown the values obtained on a group of normal rats over a five day period together with the mean values. It will be seen that there is a regular drop in the activity of the gland indicating a progressive discharge of radioiodine.

That the daily nembutal anesthesia was not influencing this effect is shown by the results recorded in Table 2. In this experiment rats 5–8

TABLE 2. SHOWING DAILY NEMBUTAL ANESTHESIA HAS NO EFFECT ON RELEASE OF IODINE FROM THE THYROID GLAND

Rat	% dose taken up at 24 hrs.	% of initial activity (24 hr. intake) on day							
		0	1	2	3	4	5	6	7
1	14.7	100	87	55	44	33	28	21	20
2	15.2	100	77	47	38	24	19	17	13
3	23.4	100	76	54	43	34	25	21	19
4	20.0	100	78	54	41	33	27	23	20
Average		100	79	53	42	31	25	21	18
5	16.0	100				32			20
6	11.4	100				34			19
7	21.8	100				29			25
8	26.2	100				29			15
Average		100				31			19

received nembutal only at the time of the initial counts and on the fifth and seventh days after; yet the decline in activity is similar to the four rats which were anesthetized daily over a seven day period.

The effect of propyl thiouracil and KSCN is shown in Fig. 1. It will be seen that the rate of decline of thyroid radioactivity is more rapid than that of the control animals.

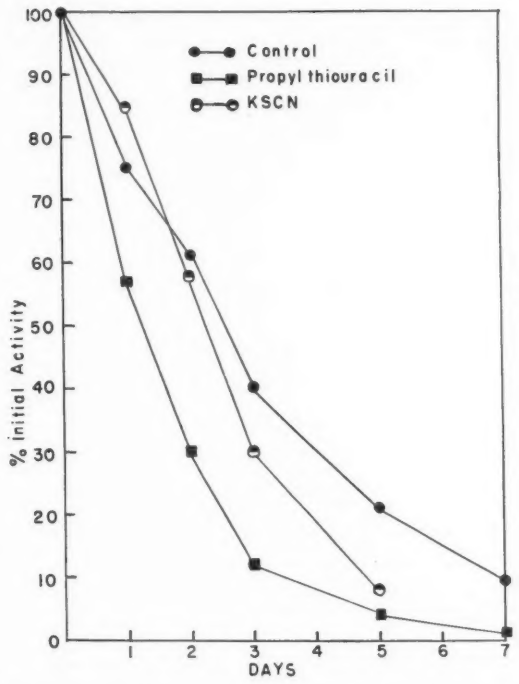

FIG. 1. The effect of propyl thiouracil and KSCN on the release of radio iodine from the thyroid.

The results of an experiment to find the effect of injections of thyrotrophic hormone is shown in Fig. 2. It will be seen that there was an increased rate of decline of thyroid radioactivity. Also shown in Fig. 2. is the effect of KI which was incorporated in the diet. This also produced a more rapid decline of the amount of iodine in the gland.

The effect of 15 μg. doses of thyroxine daily for four days is shown in Fig. 3. The values for the first five days are the average of four animals. It will be seen that the release of iodine from the thyroid was completely inhibited by doses of thyroxine of this magnitude. On the day after stop-

ping thyroxine two of the animals were given an injection of thyrotrophic hormone; this provoked an immediate release of iodine. Daily counts were continued on the remaining two rats but it was not until four days after cessation of thyroxine injections that a decline in radioactivity was noted. Thus the inhibition of iodine release produced in these rats by five daily

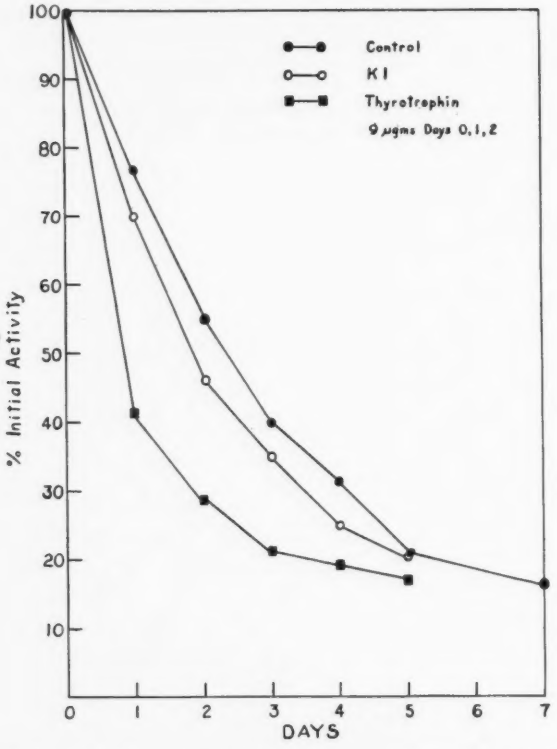

FIG. 2. The effect of thyrotrophic hormone and KI on the release of radio iodine from the thyroid.

doses of 15 µg. of thyroxine persisted for four days after discontinuing the treatment at which time the inhibition abruptly terminated.

On further investigation it became apparent that over a range of doses between 0 and 10 µg. varying degrees of inhibition of iodine release occurred with the effect being marked within two days of commencing treatment with thyroxine. These findings led to the following procedure as a means of assaying substances for their hormone activity.

METHOD OF ASSAY

Young albino male rats ranging in weight from 170–200 gm. were used for the assay. The animals were deprived of food overnight then given an intraperitoneal injection of carrier-free I^{131}. The dose used was 20–25 microcuries, all animals of any one assay being given the same dose; food

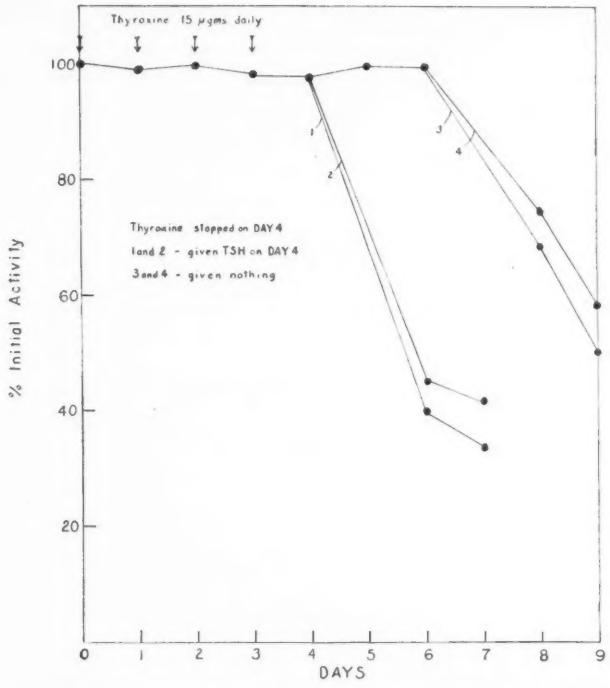

FIG. 3. The effect of thyroxine on the release of radio iodine from the thyroid.

was withheld for a further eight hours following injection of the iodine. Twenty-two hours after injection of the radioiodine the animals were given an intraperitoneal injection of 10 mgm. KSCN to discharge any inorganic iodine which might be in the gland (Vanderlaan and Vanderlaan, 1947). Two hours after the KSCN injection the animals were anesthetized with nembutal and in vivo counts over the thyroid were carried out. Following counting the animals were divided into groups, four animals to a group; and injected subcutaneously with doses of l-thyroxine ranging from 0 to 10 µg. in 0.5 cc. saline.

Groups receiving desiccated thyroid of unknown potency received

subcutaneous injections of saline and desiccated thyroid by stomach tube as an aqueous suspension of 5.0 mgm./cc. For reasons outlined in the discussion the diet was then changed from the low iodine ration to one of higher iodine content. Twenty-four hours later the above dosage schedule was repeated and twenty-four hours after this the animals were anesthetized with nembutal and again subjected to in vivo counts over the thyroid, the counting rates obtained at this time were corrected for iso-

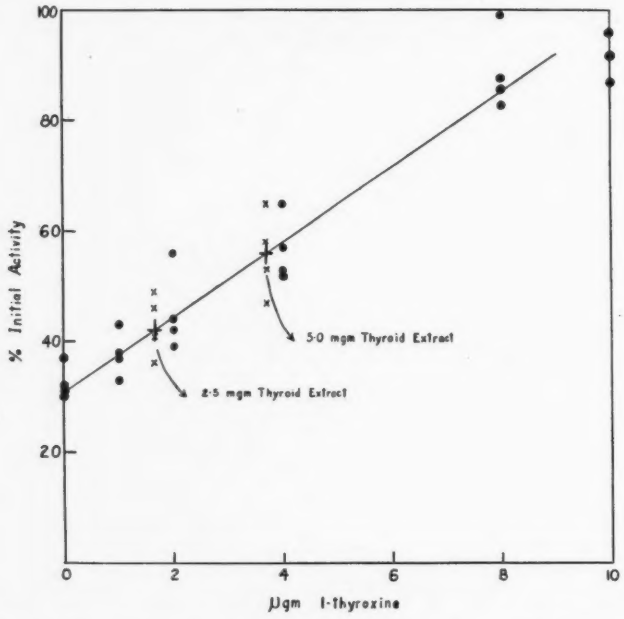

FIG. 4. The relationship between dose of thyroxine and the residual radioactivity of the thyroid.

topic decay and expressed as a per cent of the rates determined twenty-four hours after receiving the I^{131} and before receiving the thyroxine injections.

The results of such an assay are depicted in Fig. 4. It will be seen that there is a relationship between the administered dose of l-thyroxine and the quantity of radioiodine remaining in the gland. Also shown is the effect of two different doses of a preparation of desiccated thyroid of unknown potency (iodine content 0.3%). It will be noted that a dose of 2.5 mgm. had an equivalent action to 1.7 µg. of l-thyroxine while a dose of 5.0 mgm. was equivalent to 3.6 µg.

DISCUSSION

It is believed the progressive decline in radioiodine concentration of the thyroid is an index of the rate of hormone secretion by the thyroid. Support of this concept would be obtained if a change in the rate of decline of radioactivity were brought about by alterations in the amount of thyrotrophic hormone, since this factor is considered to be the chief if not the sole influence on thyroid hormone secretion (Hoskins, 1949). Thus injections of thyrotophic hormone led to a more rapid decline in thyroid radioactivity than that of untreated animals. Furthermore the goitrogens thiouracil and thiocyanate had a similar effect. These compounds, the one directly the other indirectly, interfere with hormone synthesis by the thyroid leading to a hormone deficiency; this in turn results in an augmented pituitary thyrotophin production in an attempt to compensate for the deficiency (Mackenzie and Mackenzie, 1945; Astwood *et al.*, 1943). The increased levels of thyrotophin though prevented from augmenting hormone synthesis in the goitrogen treated gland, are still able to exert their hormone mobilizing action on hormone previously synthesized and stored in the gland.

The action of KI as shown in Fig. 2. might be interpreted as an accelerated secretion of hormone as judged by the more rapid decline in radioactivity of the thyroid. However, another explanation for this is offered. As mentioned previously, since weaning the rats had been given a low iodine content diet hence ingestion of the tracer doses of I^{131} labelled a relatively small amount of stable iodine of dietary origin. On release from the gland labelled organic iodine is in due course metabolized giving rise to labelled iodide which when presented to the iodine deficient thyroid is picked up and incorporated once more into organic combination; the result would be a replacement of the lost radioactivity of the gland. Addition of KI to the diet of such animals would dilute the I^{131} and diminish its uptake and thus cause a decreased radioactivity of the thyroid. The use of a high iodine intake as described above should therefore make the decline in thyroid radioiodine more closely parallel the true rate of secretion or release of the hormone.

The administration of amounts of l-thyroxine of the order of 10 or more μg. per day results in a cessation of release of radioiodine from the thyroid, most likely a consequence of inhibition of pituitary thyrotophin secretion. This inhibition fails to a varying degree with lower doses of thyroxine and is manifest by varying rates of hormone release by the gland. This as has been shown may be made the basis of a method for assaying thyroid preparations.

From the quantities of thyroxine required to block the release of radio-

iodine it would appear that the daily requirements of a 200 gm. rat for thyroid hormone is of an activity equivalent to about 10 μg. of 1-thyroxine. This agrees with other estimates (Taurog and Chaikoff, 1947; Dempsey and Astwood, 1943).

SUMMARY

By measuring the rate of loss of radioactive iodine from the thyroid gland of the rat an index of the secretion of thyroid hormone may be obtained. The validity of this index is suggested by the effect of such influences as thyrotophic hormone, thiouracil, thiocyanate, and thyroxine.

It has been found that secretion is markedly inhibited by the administration of quantities of thyroxine of the order of 10 μg. With lower doses of thyroxine a relationship between dose and degree of inhibition of thyroid hormone secretion may be demonstrated.

Based on these facts a rapid and simple procedure for the bioassay of thyroid preparations is proposed.

Acknowledgments

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Addendum:

After the above work was submitted for publication a paper was published by J. Wolff (*Endocrinology* **48**: 284, 1951) reporting similar results.

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URINARY 17-KETOSTEROIDS IN CHRONIC LIVER DISEASE*

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ADMINISTRATION of testosterone to normal and eunuchoid human subjects has been shown to be followed by an increase in urinary 17-ketosteroids (Callow, 1939; Deakins, Friedgood and Ferrebee, 1944; Dorfman, 1940; Dorfman, 1941; Dorfman, Cook and Hamilton, 1939; Frame, Fleischmann and Wilkins, 1944; Schiller, Dorfman and Miller, 1945). All such studies have involved repeated injection of testosterone or testosterone propionate over periods of several days and determination of total neutral urinary 17-ketosteroids. It has also been reported that urinary 17-ketosteroid values are rather consistently low in patients with hepatic disease (Dohan, Richardson, Bluemle and György, 1949; Fraser, Forbes, Albright, Sulkowitch and Reifenstein, 1941; Gilder and Hoagland, 1946; Glass, Edmondson and Soll, 1940; Lloyd and Williams, 1948; Samuels, 1949), but little information is available regarding their response to administration of testosterone in such subjects (Lloyd and Williams, 1948; West, Brown, Tyler and Samuels, 1950).

The present study was undertaken for the purpose of securing data on the free and total neutral urinary 17-ketosteroids in normal subjects and in patients with chronic hepatic disease before and after a single injection of a relatively large amount of testosterone.

METHODS

Eighteen subjects (seventeen male and one female) were selected for this study, all of whom were hospitalized during the period of investigation. Their clinical status was as follows: (1) Five presented no clinical evidence of liver disease and no demonstrable impairment of liver function, four being neuropsychiatric problems and one a eunuchoid; (2) thirteen were patients with either hepatic cirrhosis or chronic hepatitis, corroborated

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TABLE 1. URINARY 17-KETOSTEROIDS BEFORE AND AFTER SINGLE INJECTION 200 MG. TESTOSTERONE PROPIONATE*

Case	Age	Condition	Serum bilirubin	BSP[1]	Urinary 17-ketosteroids[2]								% recovery
					Basal[3]	Post-injection days							
						1	2	3	4	5	6	7	
			mg.%	%									
1	21	Normal			F† 0.2 T† 13.1	0.6 39.5	0.5 34.0	0.4 26.1	0.5 17.9	0.5 12.4	0.3 17.0		0.8 40.9
2	23	Normal			F 0.2 T 12.0	0.2 35.7	0.8 28.5	0.6 17.2	0.5	0.4 12.0	0.6 10.3		1.1 26.3
3	23	Normal			F 0.2 T 12.6	0.9 18.0	0.6 26.6	0.7 18.7	0.3 12.1	0.2 10.1	0.6 11.4		1.2 12.8
4	26	Normal			F 1.9 T 11.8	2.4 41.1	3.0 40.8	2.7 26.2	1.4 15.0	2.1 13.2	4.0 12.9		2.6 59.6
5		Eunuchoid			T 4.4	19.5	23.5	15.8	10.8	13.5			
6	37	Cirrhosis	5.7		F 1.2 T 3.4	0.7 22.2	5.9 28.9	3.4 15.5	1.3 8.2	1.4 5.1	2.4 7.9	1.3 2.6	4.7 40.9
7 ♀	51	Cirrhosis	0.6	32	F 0.4 T 2.7	1.1 16.7	4.2 21.7	1.9 11.3	0.5 7.9	0.3 5.0	0.1 2.4	0.1 3.3	3.5 29.3
8		Cirrhosis			F 0.4 T 3.1	1.5 19.5	0.8 7.7	0.5 4.6	0.6 3.6	0.2 3.8	0.4 2.2		1.2 14.3
9	43	Chronic Hepatitis	1.5	48	F 2.2 T 6.0	10.9 30.1	7.1 23.0	1.4 7.2	2.0 5.4	2.0 4.6	1.8 4.7		7.2 23.8
10	34	Chronic Hepatitis	1.4	8	T 5.8	34.6		10.2	2.6	6.2	4.3	5.5	
11	22	Chronic Hepatitis	1.1	11	T 9.0	35.4	18.7	14.2	13.3	5.9	8.1	10.8	25.0
12	34	Cirrhosis	0.6	14	T 12.8	23.4	16.7	16.4	15.1	11.5	13.0	14.5	11.6
13	58	Cirrhosis	0.7	25	T 4.9	15.4	10.9	7.0	4.5	3.9	2.9	4.4	9.5
14	36	Cirrhosis	0.4	3	T 10.9	11.0	7.7	11.2	18.2	28.2	20.1	8.9	18.6
15	42	Cirrhosis	0.6	2	T 8.4	36.1	31.3	15.6		6.7	8.0	8.9	
16	39	Cirrhosis	0.4	5	T 7.0	22.3	9.9	14.3	8.7	6.3	7.9	10.3	16.4
17[4]	21	Chronic Hepatitis	0.5	11	T 9.0	13.6	18.2	11.8	8.5	8.3	6.8	6.2	~~20.6~~ 20.0
18[4]	45	Cirrhosis	0.2	6	T 4.5	9.4	13.3	8.1	5.1	4.8	5.3	4.3	22.8

* Equivalent to 167 mg. testosterone.
† "F" = Free; "T" = Total.
[1] Serum BSP 45 minutes after injection of 5 mg. per kg. body weight.
[2] In milligram equivalents dehydroisoandrosterone.
[3] Average of three pre-injection days.
[4] Received 100 mg. testosterone propionate (83 mg. testosterone).

by biopsy of the liver, with varying degrees of hepatic functional impairment on the basis of tests employed routinely (serum bilirubin concentration, bromsulfalein retention, thymol turbidity, serum albumin and globulin concentrations).

Urine was collected daily (as 24-hour specimens) for three days before and for six or seven days after a single intramuscular injection of 200 mg. testosterone propionate in sesame oil (100 mg. in two subjects with liver disease, Cases 17 and 18, Table 1).

In those cases in which free 17-ketosteroids were determined, the 24-hour specimen was divided into two equal parts, one being used for determination of free, and the other for total 17-ketosteroids.

Free 17-Ketosteroids. 75 ml. of toluene was added to one-half of the 24-hour specimen, made acid to Congo Red, and the entire sample was extracted by shaking three times with one-fifth volume of ether. The ether was evaporated and the toluene phase was extracted three times with 25 ml. 1 *N* NaOH.

The pooled toluene extracts were washed neutral to litmus with distilled water, evaporated to dryness and made up to 25 ml. with 95 per cent ethanol. Aliquots were removed, dried and subjected twice to the micro-Girard procedure. The ketonic fraction, dried from an alkali-washed ether extract, was dissolved in 95 per cent ethanol and estimated by a modified Callow procedure (Callow, Callow and Emmens, 1938) employing dehydroisoandrosterone as a standard.

Total 17-Ketosteroids. The urine sample (either entire 24-hour or one-half 24-hour) was made acid to Congo Red and was extracted three times by shaking with one-fifth volume of butanol. The pooled butanol extracts were dried in vacuo, the residue was taken up in 200 ml. H_2O and 75 ml. toluene was added. This was hydrolyzed by boiling with 15 volumes concentrated HCl for 15 minutes. The hydrolyzed material was cooled rapidly and extracted four times with one-fifth volume of ether. The pooled ether-toluene extracts were washed twice with one-fifteenth volume 9 per cent $NaHCO_3$ and once with one-thirtieth volume distilled water. The pooled extracts were heated until all ether was removed.

The toluene extract was extracted three times with 1 *N* NaOH, the neutral 17-ketosteroids remaining in the toluene phase. The subsequent procedure was as outlined for free 17-ketosteroids.

RESULTS

The pertinent data are presented in Table 1.

Normal. The total urinary 17-ketosteroids, originally within normal limits (11.8–13.1 mg.), increased to a maximum (26.6–41.1 mg.) in the first (3 cases) or second (1 case) 24-hour post-injection period, falling subsequently to reach the basal levels in 4–6 days.

The basal free urinary 17-ketosteroid excretion in three cases was 0.2 mg., increasing after administration of testosterone to maximum levels of 0.6 to 0.9 mg. in the first or second 24-hour post-injection period, falling subsequently, but tending to remain above the original level for 5–6 days, In one case (Case 4), regarded as normal clinically, the basal free urinary 17-ketosteroid value was unusually high (1.9 mg.) as were the

TABLE 2. PERCENTAGE OF URINARY 17-KETOSTEROIDS IN FREE FORM BEFORE AND AFTER TESTOSTERONE

Case	Condition	Basal	Post-injection maximum	Recovery*
		%	%	%
1	Normal	1.5	1.5	2.0
2	Normal	1.7	2.2	4.2
3	Normal	1.6	3.4	9.4
4	Normal	16.1	9.7	4.4
6	Cirrhosis	35.3	20.4	11.5
7	Cirrhosis	14.8	7.7	11.9
8	Cirrhosis	12.9	36.2	8.4
9	Chronic Hepatitis	36.6	19.4	30.2

* $\frac{\%\text{ Recovery in free form} \times 100}{\%\text{ Recovery as total 17-ketosteroid}}$

post-injection values, which fluctuated somewhat and rose to a maximum of 4 mg. on the sixth day.

The calculated recovery of the administered testosterone as 17-ketosteroid ranged from 12.8 to 59.6 per cent (av. 34.9), 0.8 to 2.6 per cent (av. 1.4) being recovered in the free state.

In Table 2 is presented the percentage of the total urinary 17-ketosteroid existing in the free state before (1.5 to 16.1 per cent) and at the peak of values after injection of testosterone (1.5 to 9.7 per cent), and also the proportion of the calculated percentage recovery represented by free 17-ketosteroid (2.0 to 9.4 per cent).

Liver Disease (*Tables 1 and 2*). The basal values for total urinary 17-ketosteroid ranged from 2.7 to 12.8 mg., being below 10 mg. in 11 of the 13 cases studied. The curve of excretion after injection of testosterone was much the same as that in the normal subjects.

The basal values for free urinary 17-ketosteroids ranged from 0.4 to 2.2 mg. in the four cases in which they were determined. The post-injection maximum ranged from 1.5 to 10.9 mg.

The calculated recovery of the exogenous testosterone as 17-ketosteroid ranged from 9.5 to 40.9 per cent (av. 21.0) in those receiving 200 mg. testosterone propionate and was 80.6 per cent in one (Case 17) of the two subjects who received 100 mg. In four cases the percentage recovered in the free form was 1.2 to 7.2 per cent (av. 4.1).

Free 17-ketosteroid comprised 12.9 to 36.6 per cent of the total before administration of testosterone and 7.7 to 36.2 per cent of the total at the post-injection peak of excretion. The proportion of the calculated percentage recovery represented by free 17-ketosteroid ranged from 8.4 to 30.2 per cent (Table 2).

DISCUSSION

Considerable interest has been evidenced in recent years in the role of the liver in the metabolism of steroid sex hormones. In vivo and in vitro studies leave little doubt of the importance of this organ in the metabolism of estrogens, and in vitro studies, particularly, suggest a similar relation to that of androgens (Samuels, 1949).

The low basal values for total urinary neutral 17-ketosteroids in the patients with chronic liver disease in this series are in agreement with previous reports (Dohan *et al.*, 1949; Fraser *et al.*, 1941; Gilder and Hoagland, 1946; Glass, Edmondson and Soll, 1940; Lloyd and Williams, 1948; Samuels, 1949). The rather consistent occurrence in such cases of an increase in total (Gilder and Hoagland, 1946; Glass *et al.*, 1940; Rupp, unpublished data) and free (Glass *et al.*, 1940; Rupp, unpublished data) urinary estrogen and a decrease in gonadotrophin (Lloyd and Williams,

1948; Zarrow, Munson and Salter, 1950; Rupp, unpublished data) suggests that the decrease in 17-ketosteroid output, which is often accompanied by testicular atrophy, is secondary to an increase in circulating free estrogen. The latter may be attributable to a decreased capacity of the liver for removing estrogen from the systemic circulation for purposes of excretion, conjugation, or other metabolic processes involved in "biological inactivation" of this hormone.

The proportion of urinary 17-ketosteroids present in the free form in four subjects with chronic liver disease in the present series (12.9 to 36.6 per cent) was much higher than in three of the four normal subjects (1.5–1.7 per cent). This seems of significance despite the high value obtained in one subject regarded as normal, particularly in view of statements in the literature, usually undocumented however, that free 17-ketosteroids are normally excreted only in very small amounts. Relatively high values for free urinary 17-ketosteroids have been reported previously in cirrhosis (Dohan *et al.*, 1949; Glass *et al.*, 1944). These data may be interpreted as indicating that the ability of the organism (presumably of the liver) to conjugate a much diminished quantity of 17-ketosteroid metabolites of endogenous testosterone is impaired.

The percentage recovery, in the form of urinary 17-ketosteroids, of a single large dose of testosterone propionate (200 mg.) was of the same order of magnitude (12.8 to 59.6 per cent) in normal subjects as has been reported (Callow, 1939; Deakins *et al.*, 1944; Dorfman, 1940; Dorfman, 1941; Dorfman, Cook and Hamilton, 1939; Frame, Fleischmann and Wilkins, 1944; Schiller, Dorfman and Miller, 1945) for quantities of 120 to 3200 mg. administered over periods of six to thirty days (14 to 70 per cent). The percentage recovery in the patients with chronic liver disease (9.5 to 40.9 per cent), although somewhat lower, was not significantly so, and, indeed, in one subject (Case 17) receiving 100 mg. testosterone propionate, the recovery was 80.6 per cent. There are very few comparable data in the literature. Lloyd and Williams (1948) observed no increase in urinary 17-ketosteroids in four patients with cirrhosis after administration of 50 mg. testosterone propionate, although there was some increase in androgenic activity. West *et al.*, (1950) found that after intravenous injection of testosterone in 25 per cent albumin solution, the blood and urine concentrations of 17-ketosteroids were lower in subjects with liver disease than in normal controls. They regarded this as indicative of decreased ability to metabolize exogenous testosterone. Our findings with regard to total 17-ketosteroid excretion are only suggestive of such a defect.

However, the post-injection free 17-ketosteroid values in the patients with liver disease (Cases 6–9) differed rather strikingly from those obtained in three of the four control subjects (Cases 1–3). We have no explanation

to offer for the peculiarly high values obtained in Case 4 (Table 1), but they deviate so markedly from those in the other three control subjects, and the basal value is so much higher than is usually found in normal individuals, that it appears advisable to exclude this case from the control group. The maximum 24-hour excretion was greater (1.5, 4.2, 5.9 and 10.9 mg., respectively, as compared to 0.6, 0.8 and 0.9 mg. in the controls) as was the percentage of the total 17-ketosteroids excreted in the free state at this time (7.7 to 36.2 per cent as compared to 1.5 to 3.4 per cent in the three controls. These data suggest impairment of the capacity for conjugating the perhaps somewhat decreased amount of 17-ketosteroids produced from exogenous testosterone. On the basis of present concepts, this defect may be regarded as a reflection of disturbance in the function of the liver in the intermediary metabolism of testosterone.

SUMMARY

The daily urinary excretion of total neutral 17-ketosteroids is considerably lower in patients with chronic liver disease than in normal subjects. The proportion excreted in the free state is increased considerably.

The calculated recovery, as urinary 17-ketosteroid, of 200 mg. testosterone propionate, administered in a single injection, was 12.8 to 59.6 per cent in normal subjects and 9.5 to 40.9 per cent in those with chronic liver disease. The proportion recovered in the free state was higher in the latter group.

These data suggest that the capacity of the organism (presumably of the liver) for conjugation of metabolites of testosterone is impaired in subjects with chronic liver disease.

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A STUDY OF THE MELANOPHORE-CONCENTRATING EFFECT OF MAMMALIAN THYROID EXTRACT IN RAINBOW TROUT*

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THE only clearly recognized function of the thyroid gland in fishes and amphibians is that of the control of metamorphosis. An actively functioning thyroid gland or its equivalent in increased concentration of thyroid hormone in the body has been shown to be necessary for the transformation of the larval into the adult stage in a number of amphibians and several teleosts. However, since not all fishes pass through a larval stage, it may be said that the role of the thyroid gland in the body economy of this class of vertebrates is still largely unknown.[1]

Recent studies by the author (Robertson 1948, 1949) on the transformation of the parr to the silvery smolt stage in rainbow trout have revealed two manifestations of increased activity of the thyroid gland in this species of fish: one is the deposition of a thick layer of guanine crystals on the scales which gives the trout a silvery appearance; the other is concentration and disintegration of the dermal melanophores in certain regions of the body. Thyroid extract or thyrotropic hormone injected into juvenile hatchery trout produced these same effects which appeared to be identical with those occurring spontaneously in the wild fish. In addition to the gradual contraction[2] of the melanophores which resulted from an increased concentration of thyroid hormone in the body, injection of thyroid extract subcutaneously or intra-muscularly was followed promptly by a localized area of pallor and intense melanophore contraction which lasted for an

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[1] Evidence for metabolic and growth-promoting functions of desiccated thyroid gland and thyroxin in fishes is largely negative in nature. (Summarized by Robertson 1948). Among amphibians only the frog has been found to exhibit increased oxygen consumption when whole thyroid gland or thyroxine were administered (Warren 1940). Schwartzback and Uhlenhuth (1936) detected an increased metabolic rate in axolotyles during metamorphosis. Thyroidectomy, while preventing metamorphosis of amphibian larvae, does not interfere with their growth (Allen 1917, Hoskins and Hoskins, 1919).

[2] The terms concentration and contraction are used interchangeably—the latter is always employed where concentration of Melanin is also used in other connotations.

hour or more. Further investigation showed that melanophores isolated from the body in skin strips behaved in the same manner (Robertson 1951).

In view of the fact that this effect of the thyroid hormone has not been previously described, it seemed worthwhile to study the phenomenon in some detail. The results of observations on the acute melanophore-concentrating action of extracts of desiccated thyroid gland and of the different constituents of the thyroid secretion and related compounds are presented in the following communication.

MATERIALS AND METHODS

Trout. Hatchery-reared rainbow trout (*Salmo gairdnerii*) were employed for the most part. A few freshly caught wild rainbow trout were also used. The conditions under which they were maintained have been described in a previous publication (Robertson (49).

Thyroid gland preparations and chemical compounds. Desiccated and finely powdered hog and sheep thyroid glands of known iodine content were provided by Armour and Company, Chicago, through the kindness of the late Dr. Fred C. Koch who supplied the author also with thyroglobulin, diiodotyrosine, iodinated casein and thyroid stimulating hormone. The thyroid powder used in the majority of the experiments contained 0.56% iodine. For thyroxine we are indebted to Mr. W. D. Sears of E. R. Squibb and Company, New York. The other compounds used, NaI, KI, NaCl, etc., were chemically pure.

The thyroid extracts were prepared by making a 2% suspension in 0.9% NaCl solution which was allowed to remain at ice box temperature for 36 hours with occasional agitation during this period. Dilutions of the clear supernatant fluid were made with 0.9% NaCl solution.

Thyroglobulin, 1% solution, was made by suspending the material in equal parts of 0.9% NaCl and n/2 NaOH, allowing it to stand in a cool place for 6–12 hours then diluting to the desired strength with 0.9% NaCl after which it was brought to the desired H-ion concentration with n/1 HCl.

Thyroxine, 1 to 10 mg. was dissolved in 1 to 2 drops of n/1 NaOH, then diluted with 0.9% NaCl to make a concentration of 1 mg./cc. To maintain thyroxine in solution it was necessary to keep the H-ion concentration at about 11/0. At pH 10.0 thyroxine began to precipitate.

Thyroid stimulating hormone, TSH, was made up in 1% solution by first triturating the powder in a small quantity of 0.9% NaCl made slightly alkaline with n/10 NaOH. Iodinated casein and diiodotyrosine were prepared in a like manner. After dilution of these solutions with 0.9% NaCl which was about pH 7.8, the reaction was at or near this point.

Skin strips. Strips of the trout's skin 0.75 to 1.0 cm. wide, extending from above the lateral line to the belly, were removed under conditions previously described (Robertson 1948). They were then placed in 0.9% NaCl solution at pH of 7.6–7.8 and kept at a temperature of 10° C. These conditions were found to be optimum for maintaining the dermal melanophores in the dispersed phase and in a highly reactive state for at least two hours (Robertson 1951). In a few experiments we employed skin strips laid epidermal side down on filter paper moistened with 0.6% NaCl and contained in small covered and cooled petri dishes.

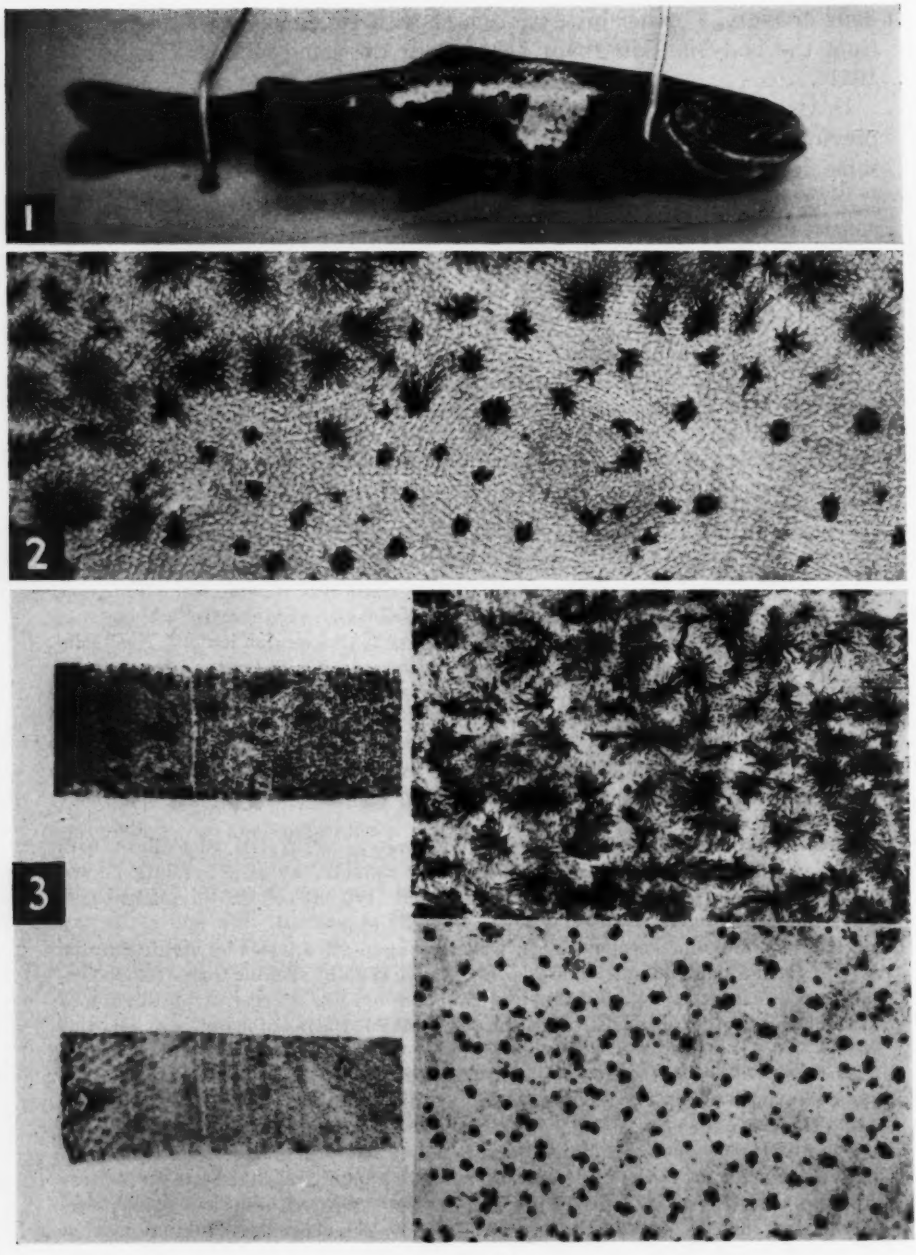

PLATE I

EXPERIMENTAL

Effect of Thyroid Extract on Melanophores in Live Trout

The subcutaneous or intramuscular injection of 0.4 cc. of a 2% extract of desiccated thyroid gland was followed within one to two minutes by beginning pallor over the site of the injection. This increased rapidly in extent and degree until usually within 4 to 5 minutes there was an area devoid of pigmentation 3 to 5 cm. in diameter, irregular in contour but sharply demarcated. Such a reaction, shown in Plate I Fig. 1. remained unchanged for 45 minutes to one hour, then began to fade and disappeared within one and one half to two hours. The only evidence of melanophore concentration other than that at the site of injection was the occasionally observed pallor spreading along the lateral line, such as shown in the photograph.[3] Individual trout exhibited considerable variation with respect to the size of the pale area and duration of the effect, probably due chiefly to differences in distribution of the injected fluid in the subcutaneous tissues. With more diluted extracts the areas of pallor were smaller, but a detectable reaction was produced with concentrations of the extract as small as 0.1%.

Examination of the reaction under the microscope revealed intense concentration of the melanophores. The margin of the pale area showing fully concentrated cells adjacent to well dispersed ones is shown in Plate I Fig. 2.

Intramuscular injection of 2% thyroid extract under the area of a parr mark produced within a few minutes pronounced concentration of the melanophores of this heavily pigmented area. These melanophores, as described in the preceding paper, react very slowly and are not affected by such influences as light and asphyxia which cause concentration of the dermal pigment cells.

[3] The lateral line appears to afford a channel for the flow of subcutaneously injected fluid. Other agents causing local concentration of melanophores have been observed to cause pallor spreading both cephalad and caudad along the lateral line region.

Explanation of Figures

Fig. 1. Sharply defined area of local pallor produced in live trout by injection of 0.4 cc. of 2% thyroid extract. Light ether anesthesia employed for photography.

Fig. 2. Photomicrograph of skin of trout at margin of reaction shown in Fig. 1. Pale area characterized by marked concentration of melanophores, while those bordering reactive zone almost fully dispersed. Magnification ×55.

Fig. 3. Two strips of skin of normal trout. Upper one, placed in 0.9% NaCl solution, remained dark and exhibited melanophores in dispersed state. Lower strip after immersion in 1% thyroid extract for 5 minutes showed marked pallor and intense concentration of the melanophores. Magnification skin strips = 1½. Melanophores = ×35.

In order to be sure that this marked local concentration of the melanophores was due to a particular property of the thyroid extract and not to the effect of protein solutions or iodine compounds, trout were injected with 1% and 2% anterior pituitary extracts, thyroid stimulating hormone and iodinated casein solutions as well as with a 1% solution of KI in 0.9% NaCl. In no instance did any of these compounds produce local or generalized pallor of the trout's skin.

Effect of Thyroid Extract on Melanophores in Isolated Skin Strips

Melanophores of isolated skin strips immersed in thyroid extract exhibited prompt and marked contraction, Plate I Fig. 3. The rate of contrac-

TABLE 1. RELATIONSHIPS OF TEMPERATURE AND CONCENTRATION OF THYROID EXTRACT TO RATE OF MELANOPHORE CONTRACTION

1. Thyroid extract 1 per cent

Temperatures of solution	Time required for contraction of melanophores*
5° C.	5 minutes
10° C.	3 minutes
20° C.	1½ minutes

2. Temperature of solutions 10° C.

Concentration of thyroid extract solutions in per cent	Time required for contraction of melanophores*
0.01 (0.1 mg./cc.)	60 minutes
0.1 (1 mg./cc.)	15 minutes
1.0 (10 mg./cc.)	3 minutes

* These are the approximate time intervals before definite beginning concentration of the melanophores was observed.

tion, observed under low power magnification, varied with both temperature and concentration of the extract. Employing a 1% extract and temperature at 5°C., contraction did not take place until 5 minutes. At 10°C. the change began at 3 minutes, while raising the temperature to 20° caused well defined contraction of the melanophores within a minute and a half. Maintaining the temperature of the solutions at 10°C. and varying the per cent concentration of the extract resulted in lengthening the reaction time with increasing dilution. These effects are shown in Table 1.

Two other factors were found to influence the rate and degree of the melanophore-contracting effect of thyroid extract. One was the H-ion concentration of the medium. For rapid and marked reactivity the optimum pH ranged from 5.0–8.0. The contracting effect of the extract was partially inhibited at pH of 10.0 and completely at 11.0 or above. The other factor

was the per cent of NaCl present in solution. With a given concentra ion of thyroid extract, contraction was most rapid with salt concentrations of 0.8% to 0.9%. The presence of 2% NaCl inhibited the reaction. In the previous study of the various factors influencing the dispersion of trout melanophores, it was found that pH above 10.0 and solutions of NaCl of 1 per cent or more caused dispersion of the melanophores. This would explain the lack of thyroid extract effect under these adverse conditions.

Concentration of the trout melanophores produced by thyroid extract was not only spontaneously reversible but could be reversed quickly by acetylcholine. These effects were demonstrable by placing drops of the solutions on the subcutaneous surface of skin strips. Within one and a

TABLE 2. THE EFFECT OF EXTRACTS FROM FOUR THYROID GLANDS VARYING IN IODINE CONTENT

Iodine content, per cent	Melanophore concentration
0.8	+++
0.62	+++
0.56	+++
0.15*	0

Note: +++ = marked concentration produced in 30 minutes by extracts of 0.1% (1 mgm./cc.).
* The sheep gland used in 0.1% to 2% extracts was without effect.

half minutes after depositing in close proximity two drops of 0.5% thyroid extract, there began to appear pallor and melanophore concentration sharply localized to the area beneath the drop. The fluid was then removed by blotting carefully with filter paper. Acetylcholine 1:100 dilution was placed on one blanched area—the other served as a control. Within one minute the melanophores under the drop of acetylcholine began to disperse, and shortly therafter the area of pallor disappeared. The control area remained unchanged for about ten minutes then gradually darkened.

Up to this point the experiments with the isolated skin strips had been carried out using the same desiccated thyroid. It was important to determine whether the melanophore-concentrating function was a common property of physiologically potent thyroid preparations, *i.e.*, those with iodine content of 0.5% or more. Tests were accordingly carried out with extracts made from three additional hog thyroid powders and one obtained from a sheep with a hyperplastic thyroid gland. The results of these tests, given in Table 2, show that desiccated hog thyroids with iodine content ranging from 0.56% to 8.0% all produced marked contraction of the skin strip melanophores, whereas the extract of the sheep gland which contained only 0.15% iodine was without effect even when used in concentrations 10 to 20 times as great as those of the hog glands.

Tests of Different Components of Thyroid Secretion and Iodine Compounds

Study of the comparative effects of thyroid extract, thyroglobulin, diiodotyrosine and thyroxine, were carried out both in the live trout and with skin strips. The results shown in Table 3 represent summarized comparisons of the size and rate of development of reactions in live trout and the rate and degree of contraction of melanophores in the skin strips. Extracts of the whole gland produced a much greater effect in lesser concentrations than did solutions of thyroglobulin and diiodotyrosine. For

TABLE 3. MELANOPHORE CONCENTRATING EFFECT OF DIFFERENT CONSTITUENTS OF THYROID SECRETION AND IODINE COMPOUNDS

Material tested	Concentration of melanophores
Desiccated thyroid (0.56% iodine)	+++
Thyroglobulin (0.46% iodine)	+
Diiodotyrosine	+
Thyroxine	0
Iodinated casein (8% iodine)	0
Sodium iodide 0.5%	0

example, it required approximately ten times more thyroglobulin than desiccated whole gland to produce the same degree of melanophore contraction. Thyroxine, even in concentrations of 0.5% mg./cc., exhibited no effect when injected subcutaneously or intramuscularly in the live trout. Its effect on skin strips could not be tested since the H-ion concentration required to hold thyroxine in solution was in the alkaline range which produced expansion of melanophores and inhibited the contracting effect of thyroid extract. In order to determine whether or not the lack of effect of thyroxine injected into the live trout was due to the alkalinity of the solution, thyroid extract adjusted to the same pH *e.g.*, 11.0, was injected in another area of the same trout receiving the thyroxine. Blanching occurred at the site of the thyroid extract injection, somewhat delayed, but after a few minutes reaching the size and degree of the reaction produced by an equivalent quantity of extract at pH 7.6–7.8. No change occurred at the site of the thyroxine injection.

Iodinated casein containing 8% iodine and sodium iodide 0.5% solution exerted no detectable effect on the dermal melanophores.

Effect of Thyroid Extract in Other Fish and One Amphibian

The melanophores of the brook trout (*Salvelinus fontinalis*) and the brown trout (*Salmo trutta*) were found to be fully as sensitive to thyroid extracts as were those of the rainbow trout. The catfish (*Ameiurus melas melas*) on the other hand exhibited no response to subcutaneous injections

of thyroid extract and very slow contraction of the melanophores in skin strips immersed in relatively strong concentrations of the extract. Melanophores of the rainbow trout showed well marked contraction within three minutes while those of the catfish skin strip immersed in the same solution began to contract only after 30 minutes. The effect on the catfish cells, however, was definite.

The melanophores of the frog (*Rana pipiens*) showed no response to thyroid extract either in vivo or in vitro. That the melanophores of the frog's skin will respond to prolonged administration of desiccated thyroid gland was shown by Warren (1940) who found that frogs fed on thyroid became pale and exhibited marked concentration of the dermal melanophores.

DISCUSSION

Relatively few references on the effect of thyroid extract or thyroxine on teleost melanophores are to be found in the literature. Wyman (1924) injected mammalian thyroid extract intramuscularly into *Fundulus* without effect on the dermal melanophores. Hewer (1925–1926) similarly obtained negative results in the minnow. Zariski (1927) fed mammalian desiccated thyroid to two teleosts, the loach, *Mignurus fossilis* and the tench, *Tinca vulgaris*, for as long as 12 weeks without any observable alteration in the state of the melanophores. A number of workers, Smith and Everett (1943), Erkin, Root and Mofshin (1940), Grobstein and Bellamy (1939) who fed desiccated thyroid and administered thyroxin to a variety of teleosts for purposes other than determining the effect on pigment cells, did not mention any changes in color occurring in the test fishes which included guppies, gold fish and *Platypoecilus maculatus* and *P. variatus*. Landgrebe (1941) on the other hand produced a striking color change in juvenile trout and salmon by injecting thyroid extract, namely transformation from the dark parr to the silvery smolt stage. He did not report any observations on the melanophores of these fishes, but in view of author's studies referred to earlier, concentration of the dermal pigment cells of the ventral surface was probably present in Landgrebe's artificially produced silvery smolts. Whether the lack of observable effect of thyroid hormone on melanophores of the other fishes cited was due to the particular species tested or to other factors such as quality or quantity of thyroid preparation employed is not apparent.

It is of interest that extracts of the whole thyroid gland were much more potent in their effect on melanophores than were solutions of compounds isolated from the thyroid, namely thyroglobulin, diiodotyrosine and thyroxine. This suggests either the presence of an unknown substance which is the principal agent in causing melanophore concentration or the

loss of some fraction from, or change in, the constitution of thyroglobulin or diiodotyrosine during their isolation from the thyroid or synthesis in vitro. The fact that thyroxine was without effect even in high concentration seems to exclude this constituent of the thyroid secretion from participation in the reaction. Likewise iodine in the form of iodinated casein and iodides of sodium and potassium had no detectable effect on the trout's melanophores. The amount of iodine in the iodinated casein and the NaI used, represents fifteen to several hundred times the iodine content of the thyroid extract, which was effective in producing marked melanophore concentration. Yet the melanophore-affecting principle of thyroid extract appears to be directly associated with the iodine content of the particular powder used since the desiccated sheep thyroid containing only 0.15% iodine (less than $\frac{1}{3}$ the iodine present in the potent preparations) exhibited no melanophore-concentrating activity.

The significance of this property of thyroid secretion for the trout has been only partially elucidated. Evidence at present available indicates that the gradual and progressive concentration of the dermal melanophores of the ventral integument of the rainbow trout, which accompanies the transformation from dark parr to silvery smolt, is caused by increased functional activity of the thyroid gland. But whether the thyroid hormone plays any role in the rapid changes exhibited by the dermal melanophores of this fish in response to various stimuli (Robertson 1951) is quite unknown. The fact that subcutaneous or intramuscular injection of high concentrations of thyroid extract cause only local and transient contraction of melanophores is not out of harmony with the possible participation of thyroid secretion in the more acute reactions of these pigment cells since adrenalin similarly injected into trout likewise produces only a local and transient effect; yet adrenalin is considered to function as one of the principal effector agents in the rapid responses of the trout's melanophores.

Parker (1940) recognizes three substances which act to control the fishes' pigmentary effector system. Two of them, adrenalin and acetylcholine, causing concentration and dispersion respectively, are produced by stimulation of the sympathetic and parasympathetic nerves supplying the pigment cells. These are the substances considered operative in the rapid alterations in the state of melanophore dispersion caused by changes in the intensity of background illumination and designated by him as neurohumors. A third substance, intermedine, secreted by the intermediate lobe of the pituitary gland causes dispersion of the melanin granules and acts more slowly than do the other two. All three of these agents were found to affect the melanophores of the rainbow trout (Rboertson 1951). Hogben and Sloane (1931) believe they have evidence for a second melanophore-affecting hormone elaborated by the anterior lobe of the pituitary

which causes their concentration. They designate this substance as "W" in contradistinction to intermedine which they call "B" hormone. Waring (1940) supported this concept in experiments on the eel as did Healey (1940) with Phoxinus. Other workers, notably Parker (1945), have failed to secure evidence for a melanophore-concentrating hormone in the pituitary of the eel or catfish. However, it seems to be generally agreed among workers in this field that the several recognized mechanisms controlling the activities of the melanophores may not all function in different species of fish (Parker 1948)—this applies particularly to the role played by the pituitary hormone or hormones. The inclusion of the thyroid hormone as another melanophore-affecting humor would provide the rainbow trout with two dispersing and two concentrating agents.

SUMMARY

Extracts of desiccated, physiologically active mammalian thyroid glands were found to cause marked and rapid concentration of the dermal melanophores of rainbow trout. Injected subcutaneously or intramuscularly such extracts produced a localized area of pallor which lasted for an hour or more. The melanophores in isolated skin strips proved to be equally sensitive to the concentrating effect of thyroid extract and provided a means for studying the optimum and limiting conditions for this reaction. Tests of the activity of the several known constituents of the thyroid secretion revealed the fact that extracts of the whole gland possessed a much greater concentrating potency than did solutions of thyroglobulin and diiodotyrosine. Thyroxine was found to have no detectable effect on the trouts' melanophores; the same was true of iodinated-casein and iodides of sodium and potassium. That the active melanophore-concentrating principle is associated with the iodine content of the thyroid, however, was shown by the absence of this property in the thyroid powder from a hyperplastic gland which contained very little iodine. The relationship of this effect of the thyroid hormone to other substances known to control activity of the melanophores in the body is discussed.

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THE EXCRETION AND DISTRIBUTION OF I^{131} FOLLOWING ADMINISTRATION OF PHYSIOLOGIC AMOUNTS OF LABELED IODIDE, DIIODOTYROSINE AND THYROXINE IN THE RAT[1]

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PRIOR to the availability of radioactive iodine, the metabolism of iodide, diiodotyrosine and thyroxine had been studied extensively in man and animals, and the pertinent literature has been excellently summarized in the monographs of Salter (1940) and Elmer (1938). These early studies were necessarily conducted by the administration of large amounts of the substances concerned in order that their fate could be followed by chemical determination of iodine. Doubt has always existed, therefore, as to whether the results so obtained with large amounts of material were characteristic also of the endogenous metabolism of these compounds. With the availability of I^{131}, similar studies have been repeated in animals on a number of occasions. Several authors have listed data showing the distribution, fate and excretion of iodide in the rat (Leblond, 1942; Perlman, Chaikoff and Morton, 1941 and Stevens, Stewart, Quinlin and Meinken, 1949). Gross and Leblond (1947) have studied the distribution of radiothyroxine at two and twenty-four hours following administration. The distribution of a large dose of radiodiiodotyrosine has been studied by Salter, Karandikar and Block (1949). However, in spite of the use of I^{131} as a labeling agent for these iodinated compounds, the specific activity of materials administered (with the exception of iodide) was far too low to be considered physiologic. It was therefore decided that there was definite need of a study of the metabolism of iodide, diiodotyrosine and thyroxine in the rat following the administration of physiologic amounts of each compound labeled with I^{131}.

MATERIALS AND METHODS

The radioactive substances were freshly prepared for each experiment.[2] In the iodide experiment, rats were injected with 1 cc. of a solution containing 0.1 microgram of

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[2] The I^{131} used in this study was obtained from the United States Atomic Energy Commission.

sodium iodide per cubic centimeter labeled with 0.5 microcurie of I^{131}. Radiothyroxine was prepared by exchange (Albert, unpublished data.) The rats in the thyroxine study were injected with 1 cc. of a solution containing 3.9 micrograms of d-1 thyroxine per cubic centimeter labeled with 1.0 microcurie I^{131}. This dose of thyroxine represents less than the daily amount of thyroxine secreted by young male rats (Dempsey and Astwood, 1943.) Radioactive diiodotyrosine was also prepared by exchange (Albert, unpublished data.) Rats used in the diiodotyrosine study were injected with 1 cc. of a solution containing not more than 6.6 micrograms of d-1 diiodotyrosine per cubic centimeter labeled with 0.64 microcurie of I^{131}. The amount of diiodotyrosine used is about half that normally present in the rat thyroid and should therefore represent a physiologic dose of the substance.

Male albino rats of the Wistar strain averaging 30 to 40 gm. in weight were used throughout the study. The animals were maintained on a commercial diet and tap water *ad libitum*. Each experiment was performed in two stages. In the first stage, simultaneous determination of the urinary and fecal excretion of I^{131} and the loss of radioactivity from the entire rat was made. Groups of 5 rats were injected subcutaneously in the nape of the neck with 1 cc. of the material to be studied. Within ten minutes of the time of injection, each rat was placed in the gamma ray counter and an *in vivo* count[3] was taken as described later. After the *in vivo* count, the rats were placed in a small metabolic cage in which the feces were collected on a fine wire mesh and the urine was allowed to drain onto heavy filter paper moistened with 4 N sodium hydroxide. At varying intervals the radioactivity of the urine (filter paper), the feces and the entire rat was determined.

The second stage consisted of determining the distribution of I^{131} in the organs of the rat. Twenty-five rats were injected subcutaneously and were divided into five groups of 5 animals each. The groups of rats were killed at varying intervals by exsanguination (aortic puncture). One cubic centimeter of blood was withdrawn for measurement of I^{131}. The following whole organs were removed from each rat: Testes, spleen, stomach and contents, large and small bowel and contents, liver, adrenals, kidneys, lungs, thymus, thyroid, brain and pituitary. A portion of the following organs was removed: Pancreas, esophagus, cardiac muscle, skin and striated muscle (gastrocnemius). All organs were weighed, placed in vials and counted separately. The carcass was also weighed and counted.

The counting equipment used consisted of a gamma ray counter (Texas Company, Houston, Texas) connected to an autoscaler (Tracer Laboratory, Inc., Boston, Massachusetts). The intrinsic variation in counting rate determined over a long period of time was less than 2 per cent. The counting efficiency of the apparatus was such that a negligible error due to the resolving time of the tube was present at a counting rate less than 1,000 counts per second, the highest counting rate employed in this study. The well of the counter (4 cm. diameter and 11 cm. depth) was large enough to accommodate an entire living, unanesthetized animal or the carcass. In counting the rat *in vivo*, the rat was coaxed into a glass tube, which was then inserted into the well.

The vials containing organs or fluids were placed in the same position in the counting

[3] In an experiment involving 42 rats, it was found that the *in vivo* count showed a mean increase in counts of 14.0 $\pm$3.7 per cent over the amount of radioactivity actually injected as determined by measurement of the syringe before and after injection of the rat. This increase in counts was probably due to scatter radiation from the bones of the rat. Consequently, the *in vivo* counts obtained ten minutes after injection were reduced by 14 per cent and the resultant value was used as 100 per cent of the dose. The same correction factor was applied to counts obtained from the rat carcass later in the study.

well. Aliquots of the dose administered were also placed in similar vials and counted simultaneously with the various organs. No count was considered significant unless it was higher than the mean background value by three or more standard deviations. The results were expressed as per cent of dose in the excretion experiments and as per cent of dose per gram tissue in the distribution experiments. Correction for physical decay of I^{131} was made when necessary, as in the excretion experiments.

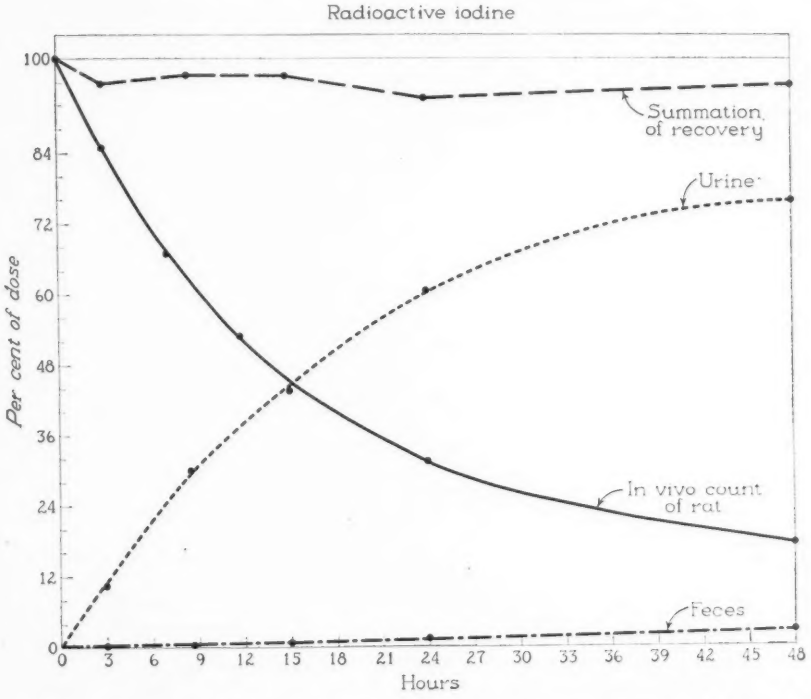

FIG. 1. Urinary and fecal excretion of I^{131} and diminution of I^{131} in the entire rat following administration of radioiodide. Each point is the average value for 5 rats, the values being plotted cumulatively. Note that at any time, *t*, the summation of the radioactivity in feces, urine and the body of the animal falls slightly short of 100 per cent of the dose.

RESULTS

1. Excretion Studies.—The excretion of I^{131} following administration of radioiodide is shown in Figure 1. Radioiodine disappeared from the entire rat in an exponential fashion. The urinary excretion of I^{131} (75.8 per cent in forty-eight hours) accounted for the major portion of the dose. The fecal excretion was negligible, being less than 3 per cent at forty-eight hours. In general these data are in excellent agreement with more extensive data

reported for human beings on the metabolism of iodide (Albert and Keating, 1949).

The urinary and fecal excretion of I^{131} following administration of radioactive diiodotyrosine (Fig. 2) was similar to that following administration of iodide from the eighth to the forty-eighth hour of the study. During the first eight hours, however, the fecal excretion amounted to 12 per cent

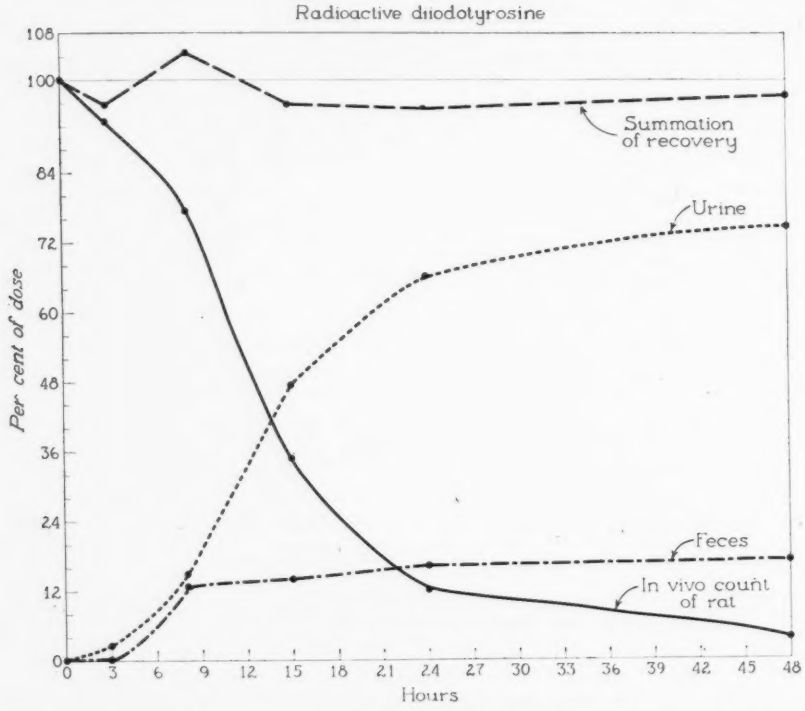

FIG. 2. Urinary and fecal excretion of I^{131} and diminution of I^{131} in the entire rat following administration of radiodiiodotyrosine. Each point represents the average value of 4 rats.

of the dose. From this time until the end of the forty-eight-hour period of observation, only a small increment of fecal excretion occurred. Unlike the curve of urinary excretion of I^{131} following iodide administration, the initial limb of the curve of urinary excretion lagged. After the eight-hour interval the curve of urinary excretion resembled that of iodide. The shapes of the excretion curves suggested that initially some of the diiodotyrosine may have been excreted as such and that after a period of eight hours the nature of the excretion had changed, resembling that of iodide.

The excretion of I^{131} following the administration of radiothyroxine was quite different from that following the administration of iodide or of diiodotyrosine (Fig. 3). A more prolonged and roughly linear diminution of radioactivity from the entire animal occurred. The urinary excretion of I^{131} was about 30 per cent of the dose at forty-eight hours, much lower than in the case of the previous substances; whereas the fecal excretion at this time was about 40 per cent of the dose, much higher than that following administration of iodide or diiodotyrosine. It was evident from the

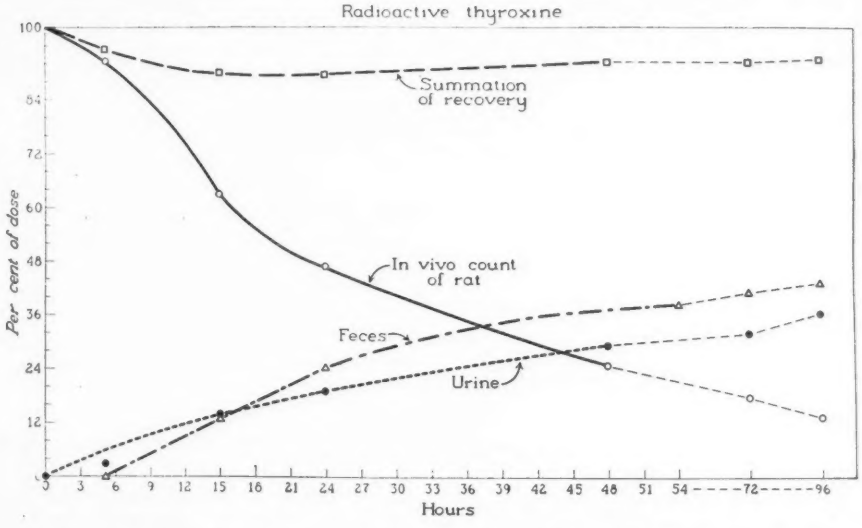

FIG. 3. Urinary and fecal excretion of I^{131} and diminution of I^{131} in the entire rat following administration of radiothyroxine. Each point represents the average value of 4 rats.

shape of the elimination curve following administration of thyroxine that the metabolism of this substance was quite different from that of iodide and of diiodotyrosine.

2. Distribution Studies.—The amount of radioactivity found in the various organs after the administration of radioiodide is shown in Figure 4. Only two organs contained more radioactivity per gram substance than did blood—the thyroid and the gastrointestinal tract. Some concentration of I^{131} was also found in the testes, spleen, pancreas, thymus, cardiac muscle, striated muscle, adrenals and brain; but the appearance of I^{131} in these organs was too sporadic and irregular and of too small magnitude to be considered significant.

Distribution of radioactivity following the administration of radio-

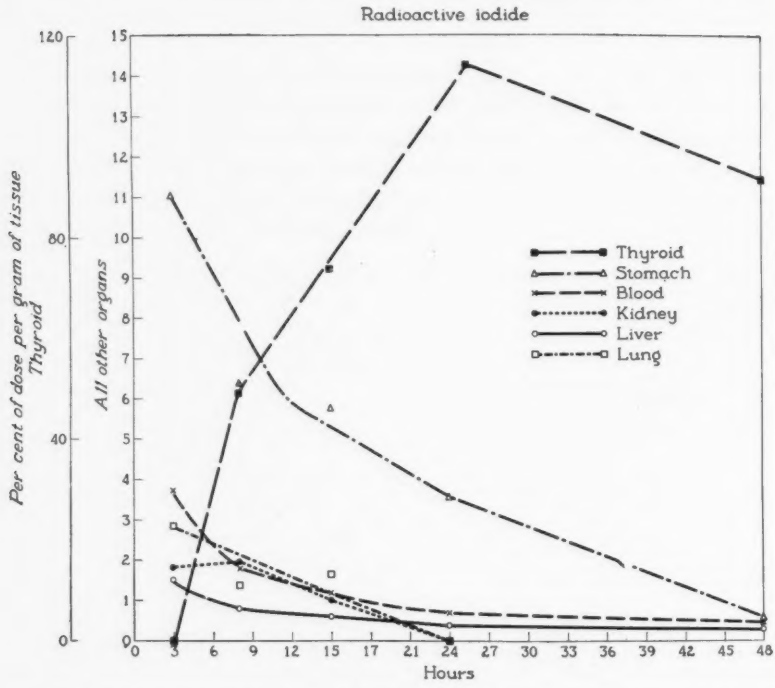

FIG. 4. Mean concentration of I^{131} in various organs of the rat following administration of radioiodide. Each point is the average value of 5 rats, and only those tissues which concentrate significant amounts of I^{131} are shown. In this graph the curve designated as "Stomach" should read "Stomach, small and large bowel." The zero value in this and in Figs. 5 and 6 represents a value three standard deviations above the mean of the background count.

diiodotyrosine is shown in Figure 5. In this instance three organs contained radioactivity in amounts greater than that present in an equal weight of blood—thyroid, stomach and kidneys. No significant concentration was found in any of the other organs.

The distribution of radioactivity following administration of radiothyroxine is shown in Figure 6. The greatest amount of radioactivity was found in the stomach. Smaller amounts but nevertheless concentrations greater than that of the blood were present in the kidneys and liver. No significant amount of radioactivity was found in the thyroid gland. I^{131} was also found in the lungs, testes and pancreas; but it occurred in too sporadic a manner and in too few animals to be considered significant. Radioactivity was not found in striated muscle, as reported by others (Gross and Leblond, 1947).

Insignificant amounts of radioactivity were present in all other tissues studied in the three experiments except in one instance. Following the administration of radiothyroxine a small amount of radioactivity was noted in the brain. However, this was much less than the amount present in an equal weight of blood.

From the foregoing experiments, it was apparent that radioactivity was concentrated in the gastro-intestinal tract after administration of iodide, diiodotyrosine and thyroxine. In order to determine more exactly the distribution of I^{131} within the gastro-intestinal tract, these experiments were repeated and separate portions of the gastro-intestinal tract were analyzed. Groups of 15 rats each were injected with labeled iodide, diiodotyrosine and thyroxine, and 5 animals were killed at three, six and twenty-four hours after injection. As standards for comparison, the radioactivity in 1 cc. of blood and in thyroid was measured. The gastro-intestinal tract

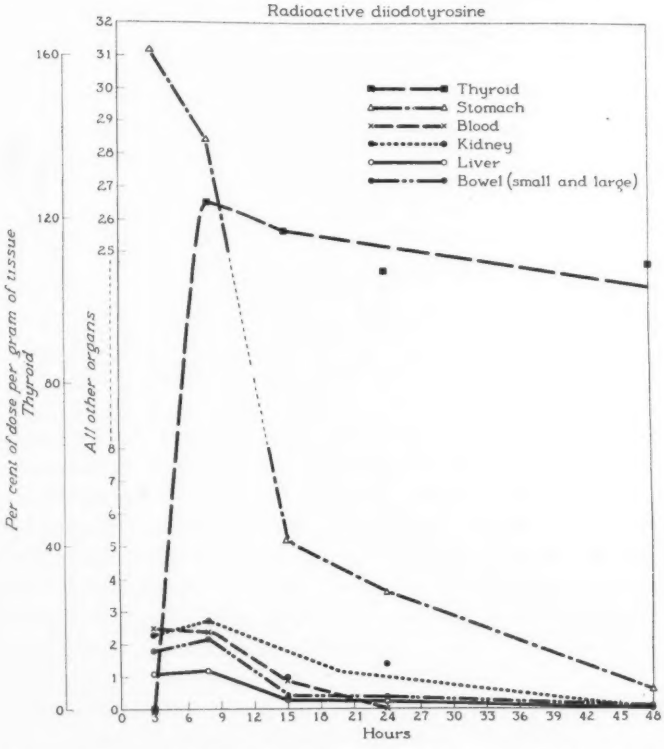

FIG. 5. Mean concentration of I^{131} in various organs of the rat following administration of radiodiiodotyrosine. Each point is the mean value for 5 rats.

from the esophagus through the rectum was removed in one piece and separated into stomach, small bowel and large bowel. The liver, pancreas and salivary gland (submaxillary) were also removed. The three portions of the gastro-intestinal tract were weighed. Each portion was then opened and the contents were allowed to drain into a flat dish. The mucosal surface was then washed with saline solution, and the washings were added to the dishes. The portions of the gastro-intestinal tract were weighed again. The difference in weight for each portion of the gastro-intestinal tract,

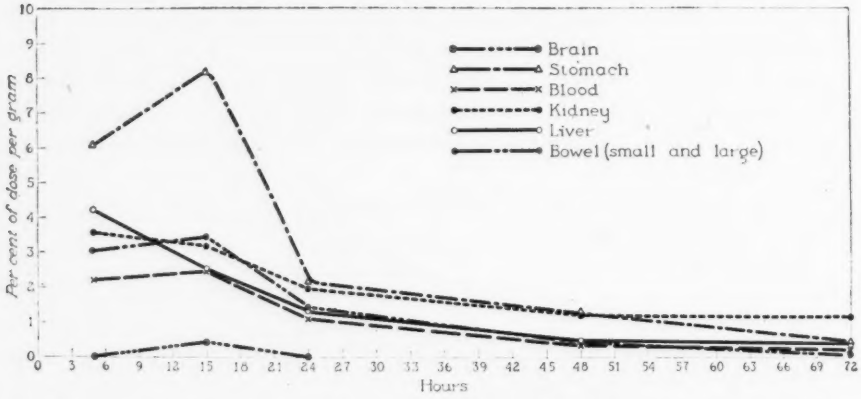

FIG. 6. Mean concentration of I^{131} in various organs of the rat following administration of radiothyroxine. Each point is the mean value for 5 rats.

before and after removal of the contents, thus represented the weight of the contents of the particular portion of the gastro-intestinal tract.

In an effort to determine something of the nature of the I^{131} present, each organ was ground with 10 per cent trichloracetic acid. After the insoluble portion had been washed with 10 per cent trichloracetic acid, the radioactivity in the precipitate was determined. The precipitate contains any free thyroxine that may be present or any I^{131} in protein-bound form. The trichloracetic-soluble portion contains both free diiodotyrosine and iodide, and the iodide can be separated by the addition of silver nitrate. Iodide is precipitated as a silver salt, leaving diiodotyrosine or similar substances in solution (Albert and Keating, unpublished data).

The results of the distribution of I^{131} within the gastro-intestinal tract are shown in Table 1. Confirmatory of the previous experience, it can be seen that after the administration of labeled iodide, localization of I^{131} occurred in the stomach and thyroid and to a lesser extent in the small bowel. However, the greatest amount of radioactivity was present in the secretions of both the stomach and small bowel. There was no concentra-

tion in the large bowel. Since the concentration tends to fall with time and thus to parallel the blood curve, it would appear that the upper part of the gastro-intestinal tract constitutes an appreciable space for iodide which is in equilibrium with the blood. The facts that the feces contain little radioactivity and that the large bowel shows no localization of I^{131} would indicate fairly complete reabsorption of iodide.

The contents of the stomach and the stomach itself, representing the highest concentration of radioactivity, were subjected to the fractionation

TABLE 1. DISTRIBUTION OF I^{131} IN VARIOUS TISSUES AND ORGANS

Tissue or organ	Per cent of dose per gram tissue								
	Labeled iodide			Labeled diiodotyrosine			Labeled thyroxine		
	Hours								
	3	6	24	3	6	24	3	6	24
Blood	2.9	2.5	0.4	1.5	1.3	0.5	1.2	0.8	0.3
Thyroid	76.9	73.0	163.9	17.7	20.2	85.2	—	—	—
Stomach	20.4	19.3	1.5	7.0	5.3	1.5	1.4	0.9	0.4
Stomach contents	102.3	156.3	4.1	35.8	95.6	5.9	6.0	16.4	3.5
Small bowel	1.7	0.7	0.2	0.6	0.6	0.2	3.8	0.8	0.2
Small bowel contents	12.0	9.2	0.7	3.5	3.3	1.0	145.8	29.7	1.0
Large bowel	1.5	0.7	0.3	0.3	0.4	0.2	0.8	0.9	0.3
Large bowel contents	1.5	1.1	0.6	1.0	1.0	0.5	4.0	59.9	2.3
Liver	1.7	1.0	0.1	0.6	0.6	0.2	4.6	4.2	2.4
Pancreas	1.8	1.7	0.6	0.6	0.8	0.3	0.7	0.3	—
Salivary gland	1.8	1.5	0.5	0.6	0.5	0.3	0.8	0.3	—

procedure previously described. It was found that 93 per cent of the radioactivity present behaved as if it were iodide, which lends further support to the thought expressed; namely, that the gastro-intestinal secretions represent simply a fairly large space for the distribution of iodide and that conversion of iodide into organic and therefore less diffusible form is not involved.

After the administration of labeled diiodotyrosine, radioactivity was found in the thyroid and upper part of the gastro-intestinal tract, qualitatively, a similar distribution as noted for iodide. Again radioactivity was present in the secretions of the stomach and small bowel and to a lesser extent within the tissue itself. Fractionation of the stomach and contents of the stomach indicated that 90 per cent of radioactivity was present in the form of iodide and not as diiodotyrosine or thyroxine. Thus, it would appear that while a small amount of diiodotyrosine may be secreted into the large bowel and thereby eliminated in the feces as such or that the thyroid may accumulate a small portion of diiodotyrosine as such, the major portion of this compound is rapidly broken down, liberating iodide. This is indicated both by the similarity to iodide in the distribution

throughout the tissues and by the rough identification as iodide of the I^{131} in the portion of the gastro-intestinal tract which concentrated most of the activity.

Distribution of I^{131} following the injection of labeled thyroxine was quite different, as noted in the previous study. No radioactivity was observed in the thyroid. A much smaller proportion was present in the stomach than after injection of iodide or diiodotyrosine. However, the small bowel and large bowel contained high concentrations of radioactivity. This would indicate that, if the radioactivity was due to thyroxine itself, this compound is poorly reabsorbed from the gut, which would explain the high percentage in the fecal excretion. Analysis of the contents of the three portions of the gastro-intestinal tract indicated that only 30 per cent of the contents of the stomach was present as thyroxine, the remainder being in the form of iodide; whereas in the contents of both the small bowel and the large bowel 90 and 96 per cent respectively of the radioactivity was present as thyroxine. It would thus appear that thyroxine is secreted into the gastro-intestinal tract, probably directly from the blood stream, that some destruction occurs in the stomach, liberating iodide, but that very little destruction or reabsorption occurs in the lower portion of the gastro-intestinal tract. That the liver, which also concentrated radioactivity, was rich in thyroxine is indicated by the fact that 82 per cent of its radioactivity behaved as if it were thyroxine. The high concentration of thyroxine in the liver taken in conjunction with the high concentration in the small bowel and the large bowel would indicate that an enterohepatic circulation of thyroxine is present and hence would explain the high distribution of thyroxine in the bowel as well as the hypothesis just offered.

DISCUSSION

In these experiments, mainly the total amount of radioactivity present in any tissue or fluid was measured and, with the exception of a few analyses on the tissues which greatly concentrated I^{131}, no attempt was made to identify the chemical nature of the I^{131}. However, it is known from previous studies (Albert and Keating, 1949) that within the time interval of these experiments, radioiodide is distributed and excreted as iodide and that no conversion into organic compounds occurs except in the thyroid. Presumably thyroxine is metabolized into at least two compounds, iodide and diiodotyrosine. Unpublished data from human experiments indicate that diiodotyrosine is rapidly decomposed and that the iodine is excreted as iodide (Albert and Keating).

The similarities and dissimilarities of distribution and excretion of I^{131} following administration of the three radioactive compounds can be noted by a comparison of the data on distribution and excretion after adminis-

tration of radioiodide with those of the radioactive organic iodine-containing compounds. If the distribution and excretion of radioactivity following the administration of organic iodine compounds were similar to that of iodide, it could be concluded that the iodide had been effectively split off. However, if radioactivity were excreted differently or concentrated in certain organs which did not collect radioiodide, it could be assumed that the organic compounds themselves or some organic metabolite had been concentrated by such tissues.

When the radioactivity in certain tissues was compared with that in the blood on an equal weight basis, it was evident that certain tissues had specific concentrating power for the three compounds. Most notable among these was the thyroid. In the iodide experiment the thyroid contained 167.9 times as much radioactivity per gram as the blood, a fact which is in agreement with a number of previous reports (Astwood, 1949; Chaikoff and Taurog, 1949). The concentrating power of the thyroid following the administration of diiodotyrosine was only one third of that in the iodide experiment, being 52 times the blood concentration. As in the iodide experiment, this high concentration of radioactivity in the thyroid after injection of diiodotyrosine was maintained even after the blood level was not measurable. The difference in urinary and fecal excretion in the iodide and diiodotyrosine experiments in the first eight hours and the early and high concentration of radioactivity in the thyroid might indicate that a small amount of diiodotyrosine itself or some other unknown breakdown product, as well as iodide split from the diiodotyrosine molecule, may have been concentrated and held by the thyroid gland. In view of the similarities of the curve of urinary and fecal excretion of I^{131} following administration of iodide and diiodotyrosine after the eight-hour interval, it would appear that the major portion of the thyroidal activity in the diiodotyrosine experiment after the eight-hour interval was due to the liberation of iodide from diiodotyrosine and its subsequent concentration by the thyroid. Significant amounts of radioactivity were not demonstrable in the thyroid following administration of thyroxine. Thus, it is obvious that no radioactive organic metabolite of thyroxine and no thyroxine itself was accumulated by the thyroid and furthermore that any iodide liberated from it was liberated at too slow a rate or in too small amounts to be effectively accumulated by the thyroid during the seventy-two hours of the experiment.

The gastro-intestinal tract concentrated I^{131} in amounts that were greater than were in the blood after administration of all three compounds. Furthermore, the secretions of the gut rather than the tissue itself contained the radioactivity. Since the radioactivity in the gastric secretion was in the form of iodide following radioiodide administration, the gastric secretion must represent a large iodide space. Furthermore, this space

is in equilibrium with the blood as shown by its decrease with time. The absence of iodide in the lower part of the gastro-intestinal tract, plus the low fecal excretion of I^{131}, after radioiodide administration, indicates that fairly complete reabsorption occurs. Such a view is confirmatory of the reports of Cohn (1932), who observed rapid absorption of iodide from isolated loops of intestine.

With the exception of the first few hours following administration of radiodiiodotyrosine, the localization of I^{131} and the nature of the I^{131} in the gastro-intestinal secretions indicate that diiodotyrosine is rapidly metabolized to iodide. Furthermore, this rapid de-iodination of diiodotyrosine would imply that because of its short biologic half-life, it plays little role as a free amino acid in either the anabolism or the catabolism of thyroid hormone.

The amounts of I^{131} in the various tissues after administration of radiothyroxine, as well as the nature of the I^{131} in the liver and gastro-intestinal tract, imply a quite different behavior in metabolism of thyroxine. This compound must be deiodinated very slowly. Furthermore, the high concentration in the liver and in the secretion of the entire gastro-intestinal tract and feces can be taken as evidence of reabsorption from the gut as well as evidence of an enterohepatic circulation. Which of these is the more important factor cannot be decided from these studies, and the question merits further direct experimentation.

Thyroxine was the only one of the three compounds concentrated by the liver. The kidneys contained greater concentrations of the three substances at the time of maximal concentration than did the blood, owing partly to the urine present in the renal parenchyma. No constant and significant uptake of radioactivity was found in striated muscle, or in pituitary or other endocrine organs, with any of the three radioactive compounds.

SUMMARY AND CONCLUSIONS

The excretion and distribution of I^{131} after the administration of physiologic amounts of labeled iodide, diiodotyrosine and thyroxine were studied in rats. The administered radioiodine and radiodiiodotyrosine were largely excreted or metabolized in forty-eight hours as measured *in vivo* by counting the entire animal. The administered radiothyroxine was lost more slowly from the body than the other two substances. The urinary excretion of radioactivity following administration of radioiodide and radiodiiodotyrosine was similar in magnitude and in time relationships except for the first eight hours. During the first eight hours, the excretion pattern indicated that a small portion of the diiodotyrosine may have been excreted as such. The curve of urinary excretion following administration of radiothyroxine was unlike that of iodide and diiodotyrosine in that it was

linear in shape and much smaller in magnitude. The fecal excretion of radioactivity in the iodide, diiodotyrosine and thyroxine experiments was 2, 16 and 40 per cent of the dose respectively.

The greatest amount of I^{131} was present in the thyroid following administration of radioiodide and radiodiiodotyrosine. No radioactivity was found in the thyroid following administration of radiothyroxine. Of the extra-thyroidal tissues the gastro-intestinal tract concentrated the greatest proportion of I^{131} administered as iodide, thyroxine or diiodotyrosine. The concentration of I^{131} was greater in the secretion of the gastro-intestinal tract than in the tissue. Following injection of labeled iodide, the I^{131} of the gastric secretion was in the form of iodide, thus constituting a rather large space for the distribution of iodide. The I^{131} in the gastro-intestinal tract following injection of radiodiiodotyrosine was largely in the form of iodide, indicating a rather rapid de-iodination of the substance. Following injection of radiothyroxine, the I^{131} was present mainly as thyroxine. The high concentration of radiothyroxine in the liver in comparison with the high fecal excretion and high concentration in the small bowel and the large bowel indicates that thyroxine probably undergoes an enterohepatic circulation or is poorly reabsorbed from the gut or both.

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THE EFFECTS OF VARIOUS STEROIDS ON THE COLLECTION OF RADIOACTIVE IODINE BY THE THYROID GLAND OF THE RAT

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INTRODUCTION

NUMEROUS reports have suggested that both the adrenal glands and the gonads might play a role in the control of normal thyroid function (Means, 1948). To our knowledge, however, no complete study, clinical or animal, has been done to determine this possible relationship. Moreover, in many cases the observations which have been made are difficult to evaluate due to great variation in experimental conditions. Gassner and co-workers (1947) and Money *et al.* (1950) have recently reviewed the literature on this subject. Therefore, further discussion will be omitted from this publication.

For some time we have been investigating the factors involved in control of normal thyroid function. In the course of our studies it was found of interest to determine the effects of various adrenal and gonadal substances on the thyroid. The criteria we have used in this investigation were (1) the effect of the compounds on thyroid weight and (2) the collection of radioactive iodine by the thyroid gland.

MATERIALS AND METHODS

Three hundred and sixty male rats of the Sprague-Dawley strain with a weight that varied between 250 and 300 grams were used in this study. Each group consisted of 10 animals which were selected for uniformity of weight and health. All animals were maintained throughout the experiment on double distilled water.

The rats in Series I to V were given a diet[1] which was low in iodine. Ten days after

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[1]

Sodium chloride	50 grams
Brewer's yeast	10 grams
Calcium Carbonate	150 grams
Wheat gluten	100 grams
Corn meal	3800 grams

TABLE 1

Compound	Source	Solvent	Total dose/animal
17α-hydroxy-11-desoxycorticosterone-21-acetate (Comp. S)	Glidden Co.	Suspension	50 mg.
Δ^4 Pregnene 17α, 20β 21-Triol-3-one 20,21-diacetate ("Triol")	Glidden Co.	Suspension	50 mg.
Allo-pregnanediol-3β 17α one-20 acetate 3 (Compound L)	Chemical Specialties Co.	Suspension	150 mg. 300 mg.
Adrenal cortex ext.	Upjohn Co.	Extract	21 cc.
Pregnenolone	Schering Corp.	Suspension	150 mg. 300 mg.
21-Acetoxy-pregnenolone	Schering Corp. and Syntex Co.	Suspension	150 mg. 300 mg.
Progesterone (Proluton)	Schering Corp.	Oil	50 mg.
Testosterone propionate (Oreton)	Schering Corp.	Oil	50 mg.
Estrone (Theelin)	Parke Davis & Co.	Oil	500 gamma
α-Estradiol benzoate (Progynon-B)	Schering Corp.	Oil	10 mg. 500 gamma
Cortisone acetate (Cortone)	Merck & Co.	Suspension	25, 50, 100 mg.
ACTH	Armour Laboratories	Saline	50 mg. (Armour std.)
Testosterone	Ayerst, McKenna & Harrison	Suspension	50 mg.
Oestriol	Ayerst, McKenna & Harrison	Oil	10 mg.
Desoxycorticosterone	Ciba	Suspension	150 mg.
Diethylstilbestrol	Squibb & Sons	Suspension	10 mg.
3,11,20 triketo, 17 hydroxy 21 acetoxy pregnane (Dihydro E)	Merck & Co.	Suspension	300 mg.
11-Dehydrocorticosterone acetate (Compound A)	Merck & Co.	Suspension	200 mg.

this diet was started, injections of the various compounds to be tested were begun. With the exception of compounds in oil, all injections were given three times a day, approximately every 8 hours. Oily solutions were given twice a day. Animals remained on this diet during the injection period of 10 days.

The animals used in Series VI were fed a diet[2] high in carbohydrates for 5 days before the injections were started. In order to determine food consumption under steroid treatment (which was not done with the low iodine diet) daily dietary intake was measured during the 10 days of injections. Steroid treatment did not significantly change food consumption in this series of animals.

Twenty-four hours before autopsy all animals were given 1 μc of carrier free I^{131} intraperitoneally. At the time of autopsy the rats were given a small dose of nembutal and exsanguinated from the dorsal aorta.

Thyroids were removed, dissected free of fat and connective tissue and weighed on a Roller-Smith balance. They were then immediately put into a 10 cc. vial containing 4 cc. of a solution of 3 parts Bouin's solution and 1 part of 1% NaI. Counting of I^{131} was done in a Texas company gamma-ray Geiger-Muller tube. The I^{131} in the thyroid tissue has been expressed as a percentage of the injected solution, which was measured at the same time and under the same conditions as the glands.

The compounds we have tested, their source, and total dosage per animal are given in Table 1.

[2] Essentially the same diet as reported by Ingle (1946). The only modification was that we used only 5 grams of cod liver oil and added 200 cc. more of water.

TABLE 2

Compound	No. animals survived	mg./ day/rat	Ave. body wt. (gms.)	Ave. thyroid wt. (mg.)	$\frac{\%\ I^{131}}{\text{Thyroid}}$	$\frac{\%\ I^{131}\ \text{Thyroid}}{\text{Body}} \times 100$
Series I						
Δ4Pregnene 17α, 20β,21-Triol-3 one 20,21, diacetate	10	5.0	266	23.3	55.6	6.4
Adrenal Extract	10	2.1 (cc.)	261	23.5	56.6	6.4
Compound S	9	5.0	269	24.6*	53.5	5.8
ACTH	10	5.0 (std.)	227*	19.9*	44.5*	5.1
Controls	8	—	269	27.1	58.9	5.9
Series II						
Cortisone	10	2.5	238*	18.6	38.8	5.0
Progesterone	10	5.0	293	23.3	53.3*	6.9*
Testosterone propionate	9	5.0	283	20.3	52.7†	7.4*
Estrone	8	50 gamma	260	20.7	57.4*	6.7*
Controls	10	—	278	21.4	41.1	5.4
Series III						
Compound L	10	15.0	284*	19.0	68.5	10.2
21-Acetoxy-pregnenolone	10	15.0	290†	19.5	66.3	10.0
Pregnenolone	10	15.0	283†	19.6	53.4	7.8
Cortisone	11	5.0	230*	17.6	45.5†	5.9*
Estradiol benzoate	9	1.0	256	20.1	62.0	8.0
Controls	9	—	267	18.0	61.3	9.1
Series IV						
Testosterone	9	5.0	316	22.7	59.4†	8.3
Estradiol benzoate	10	50 gamma	282*	23.2	45.2†	5.2*
Oestriol	10	1.0	275*	20.0†	36.8*	5.1*
Desoxycorticosterone	9	15.0	336†	26.0†	45.5	5.9*
Diethylstilbestrol	8	1.0	285*	21.0	44.0*	6.0†
Controls	10	—	321	22.5	51.0	7.4
Series V						
Cortisone	10	10.0	238*	20.6	43.3	5.0
Dihydro E	10	30.0	292	24.1†	69.2*	8.4*
Compound L	10	30.0	289	21.7	50.0	6.7
21-Acetoxy-pregnenolone	10	30.0	309†	23.9	60.8*	7.9*
Pregnenolone	10	30.0	299	22.9	61.1*	8.0*
Compound A	10	20.0	246*	17.3*	37.9*	5.4*
Controls	10	—	292	22.5	49.4	6.4
Series VI						
Cortisone	8	10.0	208*	11.6†	11.1*	1.9*
Dihydro E	9	30.0	277	15.7	29.7	5.3
21-Acetoxy-pregnenolone	8	30.0	270	15.0	26.9	4.9
Compound A	8	20.0	224†	13.7	21.3†	3.5*
Compound L	10	15.0	251	14.5	26.0	4.6*
Compound L	7	30.0	247	13.4	18.8†	3.4*
Controls	7	—	269	14.9	31.2	5.6

* $p = <0.01$.
† $p = <0.05$.

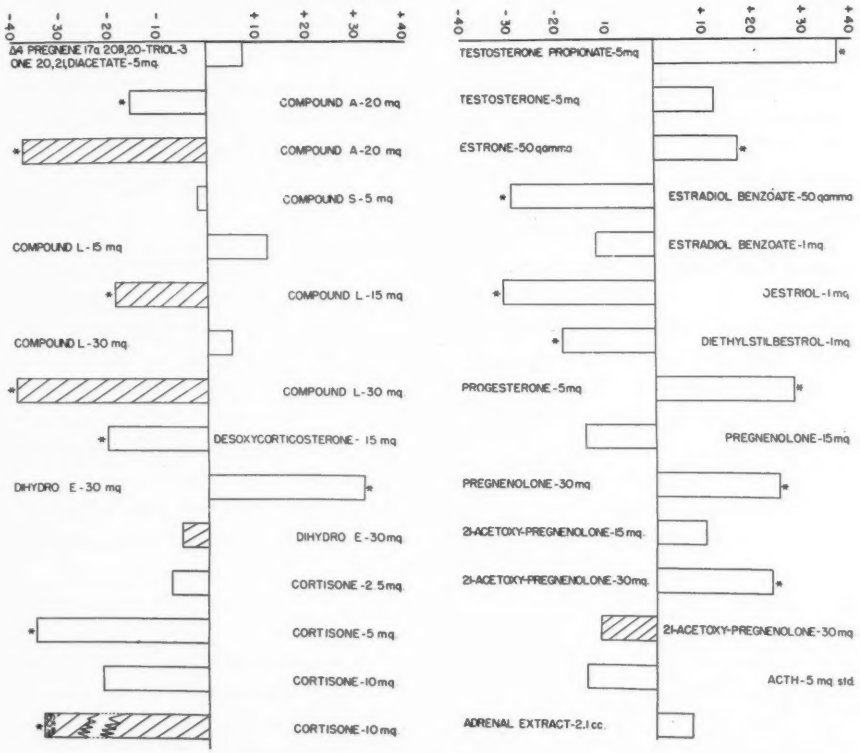

FIGURE 1 shows a comparative plot of the % I^{131} per mg. of thyroid per 100 grams of body weight (see Table 2). We have considered the control value for each experimental group arbitrarily as 100 and plotted the experimental groups as a percentage increase or decrease with respect to their controls. The center line indicates the control value. Blocks marked with diagonal lines received the high carbohydrate diet. Open blocks received a low iodine diet.

* Indicates p value of <0.05 as shown in Table 2.

EXPERIMENTAL RESULTS

The effects of our investigations with these steroids are shown in Table 2. The calculations reported are body weight following treatment, thyroid weight, per cent uptake of I^{131} per thyroid gland, and the per cent uptake of I^{131} per mg. of thyroid per 100 grams of body weight. These results represent the average for each group of animals.[3] The "p" values reported in Table 2 were obtained using the method of Fisher (1941).

[3] Studies of the steroid effect on adrenal and lymphoid tissue of these animals will be reported in a subsequent publication. Biochemical studies done on blood and other tissues of some of these animals will be reported in a future publication by Dr. Oscar Bodansky of The Memorial Center.

Figure 1 is a comparative plot of the % I^{131} per mg. of thyroid per 100 grams of body weight (from Table 2). We have considered the control value for each experimental group arbitrarily as 100 and plotted the experimental groups as a percentage increase or decrease with respect to their controls. This makes possible a comparison of different experimental groups done at different times.

I. *The effect of various steroids on body weight*

ACTH, Cortisone and Compound A all produced a marked loss in body weight of the rats in the doses used. It is interesting that these compounds when tested on seven different groups of animals produced roughly the same loss in body weight.

In the doses we have used Diethylstilbestrol, Oestriol, and Estradiol all significantly decreased body weight.

Of the compounds tested only three produced significant increases in body weight. These were 21-Acetoxy-pregnenolone, Pregnenolone and Desoxycorticosterone. The average increase in body weight was, however, only about 20 grams greater than the controls over the period of the experiment.

II. *The effect of these compounds on thyroid weight*

On the basis of absolute thyroid weight many of the steroids appear to either increase or decrease the mass of the thyroid gland. ACTH, Cortisone and Compound A apparently decreased thyroid weight, but the loss may only be a reflection of decreased body size. This is suggested by the fact that with only one exception the thyroid-body weight ratios are not significantly different from the control animals. The exception is the animals which received 5 mg. daily of Cortisone. With these rats we observed no appreciable loss in thyroid weight but an increased thyroid-body weight ratio.

Compound S significantly decreased the thyroid weight but did not shift the thyroid-body weight ratio. Desoxycorticosterone increased thyroid weight and the thyroid-body weight ratio. Another compound which induced an increase in thyroid weight but not the thyroid-body weight ratio was Dihydro E when given to animals on a low iodine diet.

III. *The collection of I^{131} by thyroids in animals treated with steroids*

A. *Compounds which produced no significant change in I^{131} uptake:* Of the compounds tested (Table 2 and Figure 1) Cortisone (2.5 mg.), Adrenal extract, Compound S and Δ^4 Pregnene 17α 20β, 21-Triol-3 one 20, 21, diacetate produced a slight but insignificant depression of the I^{131} collected. On the other hand, 21-Acetoxy-pregnenolone (15 mg.), Estradiol (1 mg.) and Compound L (low iodine intake) and Testosterone slightly increased

the collection of I^{131}. These increases are, however, not statistically significant.

In Series VI where the animals were maintained on a different diet, neither Dihydro E nor 21-Acetoxy-pregnenolone produced significant differences in I^{131} collection when the results were compared with controls.

B. *Compounds which decrease I^{131} concentration in the thyroid:* Substances which decrease I^{131} collected by the thyroid gland may do so by either reducing the amount of iodine localized or indirectly by producing a smaller gland. It is possible that both of these conditions may exist with steroid treatment depending on the compound used.

Compound S for example produced an apparent decrease in the percentage of I^{131} found in the thyroid. As can be seen from Table 2, however, this decrease may be due to a loss in thyroid weight. Neither the percentage of I^{131} per mg. of tissue, nor the amount of iodine collected in relation to the thyroid-body weight ratio is significantly different from the control value.

ACTH on the other hand reduced the iodine uptake while at the same time depressing markedly the thyroid weight. Here, however, since the body weight was lowered, the thyroid-body weight ratio was also reduced.

Cortisone in doses of 5 mg. and 10 mg. depressed iodine pickup by the thyroid. It is interesting to note that this depression was greater with the diet used in Series VI than with our low iodine diet. Compound A acted much like Cortisone and depressed the I^{131} collection. The doses used of Compound A were, however, twice the maximum amount we tested of Cortisone.

With a dose of 15 mg. of Desoxycorticosterone we observed a definite decrease in the capacity of the thyroid to concentrate radioactive iodine.

Our results with Compound L showed differences between tests done when the animals were on a low iodine intake and rats on our high carbohydrate diet. In Series VI both 15 mg. and 30 mg. of Compound L produced a definite decrease in iodine collection. Compound L given to animals on our low iodine diet seemed to slightly increase (with 30 mg.) and produce a greater increase (with 15 mg.) in the collection of I^{131}.

Pregnenolone in a dose of 15 mg. produced a decrease in the pickup of I^{131} in animals on our low iodine diet. A 30 mg. dose of this compound however, increased the I^{131} collection.

The gonadal type of steroids, Estradiol benzoate (50 μ), Oestriol, and Diethylstilbestrol all decreased the amount of iodine collected by the thyroid gland.

C. *Compounds which increased iodine collection:* Dihydro E produced a definite increase in I^{131} uptake in animals maintained on our low iodine diet. The rats in Series VI showed a slight lowering of I^{131} collection in comparison to the controls. In a dose of 30 mg. 21-Acetoxy-pregnenolone (on the low iodine diet) significantly increased the uptake of I^{131}. Although

15 mg. of this compound appeared to raise the amount of iodine collected the increase is not statistically significant. When we tested 30 mg. of 21-Acetoxy-pregnenolone on the high carbohydrate diet, we found a slight decrease in iodine uptake.

Pregnenolone, as mentioned above, in a dose of 30 mg. increased I^{131} collection whereas 15 mg. appeared to depress pickup.

Compound L in a dose of 15 mg. increased the I^{131} collection while with a higher dose the uptake, although above the control value, is only slightly so.

The gonadal steroids which produced an increase in the amount of I^{131} collected were Progesterone, Testosterone propionate and Estrone. Those compounds in the doses tested all increased iodine collection to a significant degree.

DISCUSSION

It was indicated earlier that gonadal or adrenal steroids might be involved in control of normal thyroid function. This report is an attempt to determine the effects of various steroids on the thyroid. Until such time as all the apparent adrenal and modified gonadal steroids are examined in similar studies it will be difficult to determine the exact role they play in control of the thyroid gland. The observations we have made, however, deserve further consideration.

Of the steroids biologically similar to those of gonadal origin all except Testosterone and Estradiol benzoate (in a dose of 1 mg.) produced a significant difference in iodine uptake. Progesterone, Testosterone propionate, Estrone and Pregnenolone (30 mg.) all increased iodine pickup over the control values. Estradiol (50 μ), Oestriol, Diethylstilbestrol and Pregnenolone (15 mg.) decreased the collection of I^{131}. Whether the reversal of response found with the two doses of Pregnenolone is significant from a biological standpoint is not known. The results obtained with 21-Acetoxy-pregnenolone are also somewhat difficult to interpret. We found that 15 mg. produced a slight increased uptake, whereas 30 mg. definitely increased I^{131} collection in animals receiving a low iodine diet. With the latter dose (on the high energy diet), we observed an apparent depression of I^{131} concentration, although the results are not statistically significant.

In an earlier publication (1950) we reported no effect on I^{131} uptake in animals treated with 1 mg. daily of Estradiol. These results were in agreement with those of Paschkis, Cantarow, and Peacock (1948) who found no significant difference in uptake of I^{131} in rats treated with 50 gamma of Estradiol benzoate for 1, 3½, and 4 weeks. However, when we treated our animals with 50 μ of Estradiol daily, we found a definite depression of iodine collection. This discrepancy may well be due to other possible variables between our studies and those of Paschkis *et al.*

ACTH, Compound A, Compound L, Desoxycorticosterone, Dihydro E and Cortisone were all found to alter I^{131} concentration of the thyroid. ACTH, Cortisone, Compound A and Desoxycorticosterone decreased iodine collection. However, no significant decrease was found with 2.5 mg. of Cortisone. The other compounds were not tested for the minimal effective dose to produce depression of iodine uptake.

Compound L in a 15 mg. dose increased I^{131} uptake on the low iodine diet, whereas 30 mg. produced no significant effect. Both of these doses with the high carbohydrate diet decreased iodine collection. Dihydro E when administered to animals on a low iodine intake increased the collection of I^{131}. The high carbohydrate diet with the same dose of Dihydro E reversed the response but not to a significant degree.

It is possible that certain of the differences we have found with the same compound are due to changes in dosage. It may well be that a low dose will produce an effect in one direction while a high dose will reverse the response. This throws little light, however, on the differences we have observed between animals on the low iodine diet and those on the high energy diet.

Examination of Figure 1 will show that the iodine collected by the thyroids was changed by maintaining animals on the high carbohydrate diet. This diet appears to contain some factor or factors which in conjunction with the steroids depresses I^{131} collection. Moreover, when the steroid normally produced a decreased pickup of I^{131} this diet augmented the effect of the compound. For example with Cortisone in a dose of 10 mg. plus the high energy diet the I^{131} depression was about double that observed with animals on the low iodine intake.

The same was seen with Compound A. On the other hand, 21-Acetoxypregnenolone in a dose of 30 mg. gave an increased uptake of I^{131} in animals on our low iodine diet. With the diet in Series VI and the same dose of steroid the iodine collection was reduced in comparison to the controls. The same essential picture was found with Compound L, and Dihydro E. Thus it would appear that something in this diet has the ability to alter the I^{131} collection. We are investigating further the influence of diet on I^{131} collection and utilization.

We have considered the possibility that these compounds may produce an alarm type of reaction and consequently change the ability of the thyroid to concentrate I^{131}. We have found, however, that the basic property of the thyroid to collect iodine is difficult to alter under the conditions used in this report. With the exception of the thyroid stimulating hormone and the anti-thyroid drugs few substances greatly change the amount of I^{131} which the thyroid will accumulate. We have unpublished data which shows that a non-specific substance such as formalin when given under the same conditions as our steroids must be given in a LD_{50} dose before the I^{131}

uptake is significantly changed. Therefore, it would seem that the responses we have obtained with the various steroids are probably valid and represent an effect of the administered compounds.

At the present time the mechanism of how steroids influence iodine collection by the thyroid is unknown. It is apparent that they may have a direct effect on the thyroid, or act via the anterior pituitary. On the other hand, it is well known that any change in renal excretion of iodine will affect the concentration in the thyroid. Therefore, it is quite possible that some of the results we have obtained can be explained on the basis of steroid effects on water and salt metabolism.

However, the exact mechanism of steroid action, the response of other steroids and the relation of chemical structure to biological effect remain for further study.

SUMMARY

1. The effect of various gonadal and adrenal steroids on the thyroid gland has been investigated.

2. It has been observed that ACTH and Cortisone decreases the accumulation of I^{131} by the thyroid. Other compounds which act in a similar manner are Compound A, Desoxycorticosterone, and certain estrogenic substances.

3. Testosterone, Estrone, Progesterone, and some other compounds appear to increase the collection of I^{131} by the thyroid.

4. Dietary differences apparently may reverse the effect produced by steroid treatment or may augment the response.

Acknowledgment

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INFLUENCE OF STRESS STIMULI ON LYMPHATIC TISSUE OF ADRENALECTOMIZED MICE

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INTRODUCTION

THERE is ample verification that the acute involution of lymphatic tissue associated with most stresses is a result of increased secretions of adrenal cortical hormones caused by stress stimuli (Dougherty and White, 1944, 1945, 1947; Yoffey and Baxter, 1946; Ingle, 1950; Yoffey, 1950). However, it should be considered that stresses (defined here as stimuli which activate the mechanisms resulting in increased adrenocortical secretion) might also alter lymphatic tissue by mechanisms other than mediated by adrenocortical hormones. Responses of lymphatic tissue to such an-adrenocortical influences of stress stimuli would be obscured in intact animals by the lymphocytolytic effect of the stress-produced adrenocortical hormone output. Therefore, the occurrence of an-adrenocortical responses in the complex of lymphatic tissue stress reactions can only be determined in animals deprived of their adrenal cortex. Accordingly, various reactions of lymphatic tissue were studied in adrenalectomized mice subjected to different stress stimuli (fasting, histamine and anaphylactic shock) which were previously shown to produce acute lymphatic tissue involution in intact animals (White and Dougherty, 1947; Dougherty and White, 1947; Dougherty 1950).

MATERIALS AND METHODS

Six hundred and forty-five CBA mice of both sexes between 12 and 16 weeks of age were used in these studies. With the exception of the fasted group, the animals were fed *ad libitum* on dog chow (Purina), supplemented with calf meal (Larro) before and during the experimental periods. Animals were maintained in quarters kept at a constant temperature. The mice subjected to fasting were kept in individual containers with screen bottoms to prevent coprophagy and received water *ad libitum*.

Tail vein blood was used for all counts. Differential counts were performed on blood

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films stained with May-Grünwald-Giemsa, at least 200 cells being differentially counted in each film. Lymph node weights from each animal were obtained from weighing the totality of inguinal, axillary and mesenteric lymph nodes. Weights of thymus and spleen were determined separately. The organs were fixed in Zenker's solution and stained with hematoxylin-eosin or methyl green pyronin for histological study.

Bilateral adrenalectomy was performed by the usual posterior approach. When animals were also splenectomized, both extirpations were made at the same time.

TABLE 1. ALTERATIONS IN LEUCOCYTE VALUES FOLLOWING ADRENALECTOMY ALONE AND SUBJECTION OF ADRENALECTOMIZED ANIMALS TO STRESS STIMULI

Groups and treatment	Number of animals	Hours after adrenalectomy	W.B.C., thousandths per cmm.³	Polymorphonuclears, thousandths per cmm.³	Lymphocytes, thousandths per cmm.³	Eosinophils, per cmm.³
			Mean S.E.	Mean S.E.	Mean S.E.	Mean S.E.
Intact controls	150	0	12.58±0.27	3.87±0.12	8.60±0.23	117± 8
Adrenalectomized non-stressed controls	11	1	12.92±0.32	7.47±0.80	5.54±0.51	32± 8
	7	2	10.66±1.54	3.78±0.89	6.86±1.26	13± 13
	11	3	11.21±1.41	3.66±0.82	7.55±0.74	0
	12	4	15.22±1.32	4.54±0.57	10.62±0.96	55± 25
	7	5	11.02±1.54	2.06±0.45	8.88±1.41	26± 18
	11	6	11.87±1.02	4.29±0.72	7.57±0.55	14± 10
	7	7	11.30±1.08	2.22±0.27	9.03±1.14	51± 28
	7	8	10.70±0.94	3.32±0.68	7.33±0.85	52± 27
	13	12	12.85±0.60	4.38±0.48	8.39±0.52	67± 25
	16	24	23.15±3.29	7.06±1.31	15.95±2.04	117± 41
	10	48	17.98±1.55	3.43±0.93	14.49±1.14	63± 27
	10	96	19.28±1.48	3.78±0.46	15.27±1.42	242± 69
	10	144	22.04±1.21	7.12±0.78	14.56±0.87	357± 52
Intact fasted 48 hours	19	0	13.94±0.87	8.55±0.79	5.36±0.49	34± 14
Adrenalectomized fasted*	5	1	22.45±1.49	14.23±1.60	8.22±1.64	41± 41
	10	2	20.17±0.68	8.38±1.11	11.76±1.46	22± 22
	5	3	20.57±1.31	7.65±1.19	12.72±0.78	183±136
	5	4	21.82±1.91	3.30±0.77	18.48±1.80	44± 43
	14	6	21.51±1.89	6.19±0.92	14.38±4.24	299± 69
	5	8	26.68±1.93	4.88±0.44	21.60±1.72	187± 76
	9	12	23.53±2.47	3.99±0.81	19.61±2.05	15± 15
	9	24	17.14±1.95	2.29±0.42	14.83±2.32	28± 19
	13	48	24.05±7.06	6.93±1.19	17.08±3.60	43± 29
Adrenalectomized given 750 mg./kg. histamine subcutaneously	9	4	22.69±1.98	10.77±1.43	11.69±1.68	234± 45
	8	8	26.66±3.49	12.12±1.23	14.21±2.52	328± 74
	7	12	37.49±4.95	13.01±2.56	24.24±3.59	160± 64
	7	24	22.16±2.70	5.59±0.94	16.41±1.91	155± 46
Adrenalectomized anaphylaxis	26	4	20.24±1.26	7.68±0.81	12.42±0.72	140± 36
	25	8	44.98±3.64	17.81±1.25	26.75±2.02	446± 95
	9	12	30.82±1.81	11.19±1.88	19.47±2.02	160± 71
Adrenalectomized splenectomized anaphylaxis	10	4	34.63±3.44	15.89±2.28	18.55±1.92	181± 63
	9	8	34.87±4.15	17.46±2.79	17.19±1.98	218± 93

* Adrenalectomized after having been fasted for 48 hours.

Two groups of animals served as controls. The first group consisted of non-treated intact mice. Control values for total and differential leucocyte counts were established in 150 non-treated intact CBA mice. Lymphatic organ weights were determined on other intact mice of the same age. The second control group was made up of non-stressed adrenalectomized mice of the same age and sex as those used in the stress experiments. Leucocyte values and lymphatic organ weights were obtained on these animals at various intervals after adrenalectomy as specified in Tables 1 and 2. In a third group, serving as control for the fasted adrenalectomized group, intact animals were subjected to starvation.

The experimental animals consisted of adrenalectomized mice which were divided into 4 groups according to the type of stress stimulus used. The first group comprised mice subjected to fasting. The animals were fasted for a 48-hour period both before and

after adrenalectomy. A second group of mice was given a single subcutaneous dose of 750 mg./kg. of histamine diphosphate dissolved in 0.25 cc. of physiological saline 2 hours after adrenalectomy. A third group of animals was composed of mice subjected to anaphylaxis. These animals were sensitized by administration of 1.2 cc. of horse serum 21 days before the challenging dose. They were challenged 2 hours after adrenalectomy by intravenous administration of 0.001 cc. of horse serum per 20 gm. of mouse. This amount of antigen had been established previously as the LD_{70}. The last group of experimental animals were treated exactly as the previous group except that they were splenectomized as well as adrenalectomized.

Blood counts were performed on the experimental animals at various intervals after stress (Tables 1 and 2). The weights of the lymphatic organs of mice subjected to anaphylaxis were obtained 4 and 8 hours after adrenalectomy.

TABLE 2. INFLUENCE OF STRESS STIMULI ON THE WEIGHT OF LYMPHATIC ORGANS OF ADRENALECTOMIZED ANIMALS*

Treatment	Hours after adrenalectomy	Number of animals	Age in weeks	Body weight	Lymph node weights	P† values	Pooled‡ Lymphatic organ weights	P values	Spleen	P values
				gm.	mg.		mg.		mg.	
Intact controls	—	24	14	21.9±0.8	184±11	—	356±11	—	330±15	—
Adrenalectomized non-stressed	4	8	14	23.8±0.8	170±13	>.05	329±22	>.05	357±16	>.05
Adrenalectomized non-stressed	8	8	14	21.8±0.9	203± 9	>.05	398±25	>.05	342±16	>.05
Intact controls	—	25	16	22.4±0.6	169± 6	—	322±10	—	318±22	—
Adrenalectomized anaphylaxis	4	27	16	24.1±0.8	211± 8	<.01	386±12	<.01	450±32	<.01
Adrenalectomized anaphylaxis	8	27	16	24.3±0.9	218±10	<.01	361±18	>.05	441±26	<.01

* Organ weights are expressed in terms of 100 gm. body weight of intact controls of the same age. Values are means and standard errors.
† P values are comparisons of means of experimental groups and intact controls of the same age. Values <.05 considered statistically significant.
‡ Combined weights of lymph nodes and thymi.

RESULTS

Alterations in the leucocyte pattern of non-stressed mice following adrenalectomy. It is evident from the data shown in Table 1 that there was no alteration in the leucocyte count of non-stressed animals following adrenalectomy until the 24th hour at which time a significant leucocytosis appeared. After this time a leucocytosis was constantly present during a subsequent 5 day period of study. The absolute number of polymorphonuclears did not follow any constant trend after adrenalectomy. Their number was significantly increased one hour after operation but was again within normal limits in most of the later samples. The absolute number of lymphocytes, on the other hand, after an initial drop at one hour rose sharply at 24 hours and maintained this level for the remaining period of study (fed animals, Fig. 1). An eosinopenia which, however, was not significant at all intervals, was present in the period between 1 and 12 hours after operation.

Effect of fasting on leucocytes of adrenalectomized mice. Intact mice fasted for 48 hours had normal total leucocyte count, polymorphonuclear leuco-

cytosis, eosinopenia and lymphopenia (Table 1, Fig. 1). One hour after these mice were adrenalectomized the total leucocyte count increased and this leucocytosis was maintained during the subsequent period of study (Table 1). The absolute number of polymorphonuclear leucocytes increased to a very high level one hour following adrenalectomy but returned to a normal value at 4 hours and thereafter tended to remain within normal limits. The eosinopenia of the pre-adrenalectomy fasting period remained

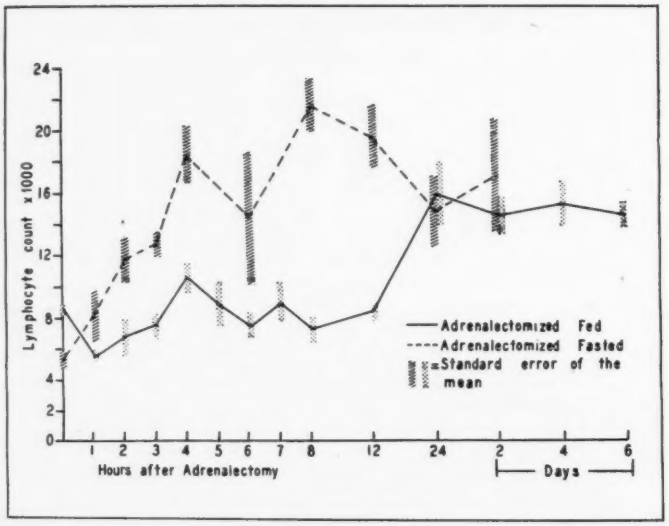


FIG. 1. Comparison of numbers of lymphocytes of fasted and fed mice at various time intervals after adrenalectomy. The value for the number of lymphocytes plotted at zero time for fed animals was determined on 150 mice; that for the fasted animals on 19 mice starved for 48 hours prior to adrenalectomy.

during the first 2 hours following adrenalectomy after which time the count rose toward normal values. The absolute number of lymphocytes rose to normal within the first hour and continued to rise until a high peak was reached 8 hours after adrenalectomy (Fig. 1). At the 24th hour following adrenalectomy, the number of lymphocytes had returned to the moderately increased value characteristic for non-fasted adrenalectomized mice.

Effect of administration of histamine on leucocytes of adrenalectomized mice. Within 2 hours after administration of histamine (4 hours after adrenalectomy) the total leucocyte count was almost doubled and remained high during the subsequent 24-hour period of observation (Table 1). An absolute increase of polymorphonuclears and eosinophils was observed at 4 hours and at all subsequent sampling times (Table 1). A rise in the

number of lymphocytes began 2 hours after administration of histamine (4 hours after operation), became statistically significant after 8 hours and reached its peak after 12 hours; the values had returned after 24 hours to the number found in non-stressed adrenalectomized mice (Fig. 2). A similar rise in lymphocytes was observed when the histamine was given as late as 6 days after adrenalectomy.

Effect of anaphylactic shock on leucocytes and lymphatic organs of sensi-

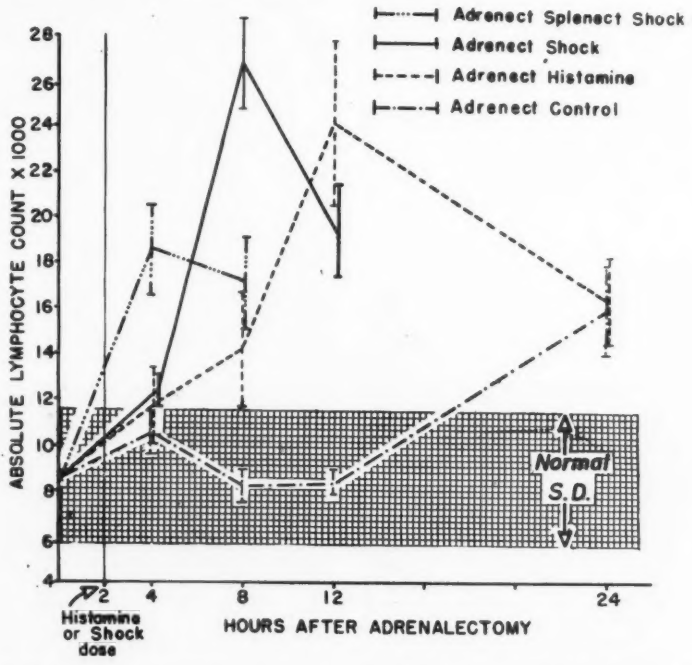
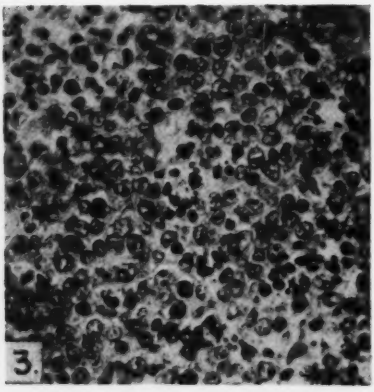


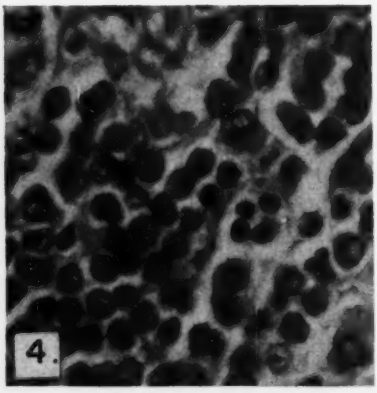
FIG. 2. Comparison of lymphocyte counts of adrenalectomized and adrenalectomized splenectomized mice subjected to sublethal anaphylaxis or administration of histamine to lymphocyte counts of non-stressed adrenalectomized mice. The value plotted at zero time is the mean number of lymphocytes determined on 150 CBA mice 14 to 16 weeks of age; the cross hatched area represents one standard deviation from this mean. The vertical bars indicate the standard error of the mean value at each time interval.

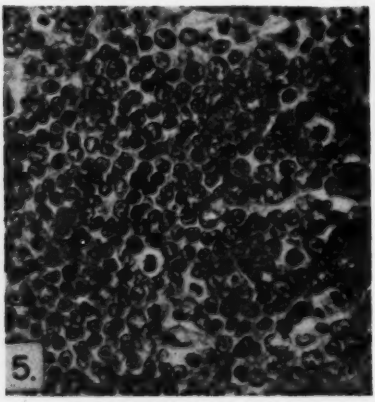
tized adrenalectomized mice. The alterations in the total leucocyte count of animals subjected to anaphylactic shock were more pronounced than after the other stress stimuli (Table 1). The number of polymorphonuclear leucocytes was increased after 4 hours but did not reach its peak until 8 hours following operation. A significant eosinophilia was present 8 hours after adrenalectomy. The number of lymphocytes increased precipitously

within 2 hours after administration of the shock dose (4 hours after adrenalectomy) and reached an extremely high value 8 hours following adrenalectomy (Fig. 2). Twelve hours after operation (10 hours after anaphylactic shock) the lymphocytes were back within the range characteristically found in adrenalectomized non-stressed animals after 24 hours.

It is apparent from the data shown in Table 1 and Fig. 2 that the absence







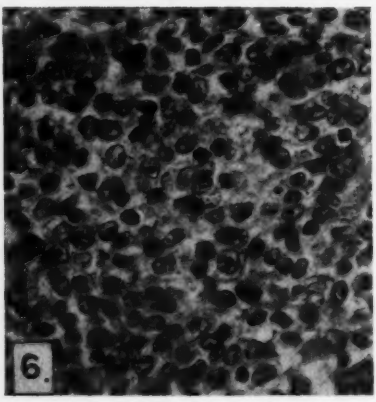


FIG. 3. Mesenteric lymph node of sensitized intact mouse 45 minutes after administration of challenging dose of horse serum. Although lymphocytolysis is extensive there is little phagocytosis of the nuclear debris. (×450).

FIG. 4. Thymus of sensitized mouse treated as in Fig. 3. Lymphocytolysis and edema are found throughout the cortex (×900).

FIG. 5. Mesenteric lymph node of adrenalectomized mouse in anaphylactic shock (45 minutes after challenging dose, 2 hours and 45 minutes after adrenalectomy). Lymphocytolysis is absent. Numerous mitotic figures are found throughout the node. (×450).

FIG. 6. Thymus of adrenalectomized mouse treated as in Fig. 5. There is a complete absence of lymphocytolysis and edema. Mitotic figures are numerous. (×450).

of the spleen did not modify the general trend of changes in leucocyte values induced by anaphylactic shock in adrenalectomized animals. The total leucocyte count and the absolute numbers of polymorphonuclears and lymphocytes were greater at 4 hours than in any of the other groups of stressed adrenalectomized animals (Table 2, Fig. 2). However, the number of lymphocytes in this group of animals did not continue to increase after the 4th hour as it did in the non-splenectomized mice. The number of eosinophils did not vary significantly from that found in intact mice.

The lymphatic organs and spleens of non-stressed adrenalectomized mice did not change in weight during the period of observation (Table 2). However, adrenalectomized animals subjected to anaphylactic shock had significantly greater lymph nodes and pooled lymphatic organ weights 2 hours after administration of the challenging dose of antigen (4 hours after adrenalectomy). The lymph nodes were still significantly larger after the 8th hour but the thymi were smaller than those of intact controls. This decrease in thymus weight accounts for the decrease in weight of the pooled lymphatic organs seen at 8 hours. The spleens of these mice weighed more at both 4 and 8 hours than those of non-stressed adrenalectomized animals (Table 2).

Morphological alterations in the lymphatic organs of both sensitized intact and adrenalectomized animals subjected to anaphylactic shock were manifest within 45 minutes after administration of the challenging dose. Extensive lymphocytolysis took place in lymph nodes, spleens and thymi of the sensitized intact mice (Figs. 3 and 4). The lymphatic organs of the challenged sensitized mice failed to exhibit lymphocytolysis. An increased number of lymphocytes in mitosis was observed in these animals (Figs. 5 and 6). Similar findings were observed in adrenalectomized fasted groups of animals (data to be published elsewhere).

DISCUSSION

An analysis of the results reveals that the increase in total leucocyte count of non-stressed adrenalectomized animals seen 24 hours after operation was due to the absolute increase in the number of lymphocytes. The transient polymorphonuclear leucocytosis, lymphopenia and eosinopenia which occurred one hour after adrenalectomy are to be attributed to the stress of the operative procedure. The fact that the number of blood lymphocytes did not increase continuously but remained at the level found 24 hours after adrenalectomy suggests that there are mechanisms other than those mediated by secretions of the adrenal cortex which regulate proliferation of lymphocytes.

The stress stimuli employed in this study modified the time of appearance and the extent of the alterations which occur in lymphatic tissue as

a result of adrenalectomy. The lymphocytosis was greater in animals subjected to stress stimuli, appeared earlier, rose to higher levels and returned 24 hours after operation to the level characteristic of non-stressed adrenalectomized animals. This lymphocytotic response is an inverse image of the "lymphopenic response" (Dougherty and White, 1947), induced by stress stimuli in intact animals. The lymphatic organs of animals subjected to anaphylaxis increased in weight more rapidly than the organs of non-stressed adrenalectomized animals. The thymi of these stressed animals, however, after an initial increase declined in weight so that 6 hours after anaphylactic shock they were smaller than those of intact animals of the same age.

Histological examination of the lymphatic organs revealed that the lymphocytolysis observed in intact animals subjected to anaphylaxis was absent in the lymphatic organs of the stressed adrenalectomized animals. That the number of mitotic figures in the lymphatic organs is higher in stressed than in non-stressed animals indicates that the rate of lymphocytopoiesis is enhanced after stress.

From the above findings it is thus apparent that all the different stress stimuli employed in this study are capable of producing reactions in lymphatic tissue which are not mediated by adrenal cortical hormones. The major stress response in the absence of the adrenal is a greater and more rapid increase in the amount of lymphatic tissue than occurs in non-stressed adrenalectomized animals. That the evacuation of lymphocytes from the spleen plays no major role in these stress responses is further indicated by the observations that lymphocytosis occurred in splenectomized adrenalectomized animals in anaphylaxis and that in non-splenectomized animals the spleen weight increased rather than decreased.

Major attention, therefore, in the search for an explanation of the an-adrenocortical stress response is directed to the enhanced lymphocytopoiesis here demonstrated. The lymphatic tissue proliferation is due to as yet unknown factors. Their influence may be actively augmented by stress stimuli or the stress stimuli may permit them more unrestrained action by removing antagonistic growth inhibiting influences. An analysis of these factors involved in lymphopoiesis may open up a deeper understanding of lymphatic tissue function as well as the exploration of the special phenomenon reported here.

SUMMARY

Time relationship and extent of alterations in lymphatic organ size and circulating lymphocytes were compared in stressed and non-stressed adrenalectomized CBA mice. Stress stimuli were starvation, histamine and anaphylaxis. Animals stressed 2 hours after adrenalectomy had a signifi-

cant lymphocytosis 2 hours later, which rose to enormous levels at 8 and 12 hours and returned to the level found in adrenalectomized non-stressed animals at 24 hours. This "lymphocytotic response" is an inverse image of the lymphopenic response of intact animals. After adrenlectomy, lymphatic organs of mice in sublethal anaphylaxis were significantly larger than those of non-stressed animals at comparable time intervals. Lymphocytosis of stress occurred also in splenectomized adrenalectomized mice and, hence, was not due to evacuation of lymphocytes from the splenic reservoir.

The observations show that adrenalectomy exposed heretofore unknown stress effects which are not mediated by adrenocortical hormone. These influences are compatible with the concept that the stimulus of stress not only augments pituitary adrenocortical secretion which inhibits lymphatic tissue growth, but also augments, directly or indirectly, lymphatic tissue growth promoting influences unrelated to adrenocortical secretion.

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STEADY STATE OF THE THYROIDAL IODINE

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FINDINGS of tracer work in biology are generally interpreted on the assumption that a "steady state" of the chemical processes in organs and tissues exists. An organ or tissue is said to be in a "steady state" with respect to one particular substance or metabolite when the amount of this substance or metabolite remains constant and, therefore, its rate of appearance in the organ or tissue is equal to its rate of disappearance. Thus, work with radio-iodine is generally interpreted on the assumption that the entry of iodine into the thyroid gland and its release as hormonal iodine take place at the same rate. The iodine content of the gland would thus remain unchanged. However, such a "steady state" of the thyroidal iodine has never been demonstrated.

An example will illustrate the importance of knowing whether or not the thyroid gland is in such a "steady state." Radioautographs of rat thyroids one hour after injection of radio-iodine showed the presence of labeled thyroglobulin in the apex of the thyroid cells, while 24 hours after injection the labeled thyroglobulin was chiefly in the colloid (Leblond and Gross, 1948). On the assumption of a "steady state" it could be concluded from the above observations that each thyroid cell was at all times synthesizing some thyroglobulin and excreting some into the colloid. The results showed that at the time of observation, there was no excretion of thyroglobulin in the reverse direction, i.e., into the circulation. If, on the other hand, there is no "steady state," the cells might at times stop functioning or excrete directly into the circulation, in which case the thyroid activity would be cyclic. It is, therefore, apparent that the above experiment would lend itself to a far broader interpretation if a "steady state" of thyroidal iodine could be demonstrated. In other problems, such as the calculation of the turnover rates of the various chemical components of the thyroid gland, the available method (Zilversmit *et al.*, 1947) requires the existence of a "steady state," and no conclusions at all can be reached in its absence.

The present paper is an attempt to demonstrate the existence of a "steady state" of thyroidal iodine. It was decided to examine this problem by investigating the iodine metabolism of the thyroid at frequent intervals

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over a 24-hour period. Each such period includes physiological changes due to the intake of food at definite times and to the sequence of rest and wakefulness. If a "steady state" were to exist over that time, it would be likely to extend to successive, similar 24-hour periods.

To establish that the thyroidal iodine is in a "steady state" over a 24-hour period, the first step was to demonstrate that the iodine content did not change in that time interval. However, this method did not provide a sensitive index of fluctuations occurring over short periods of time nor could important changes in the chemical nature of iodine be detected.

It was, therefore, necessary to investigate several of the phases of thyroid activity. Three problems were considered. Firstly, are the thyroid activities performed continuously or intermittently? Secondly, do some of the activities (e.g. iodine fixation) alternate with others (e.g. release of iodine products)? And thirdly, does the rate at which each or all such activities proceed vary or is it constant? Two thyroid activities were investigated, namely: iodine fixation and hormone secretion by the gland as measured by the protein bound iodine of the plasma. This was done by working at six equal time intervals taken throughout a 24 hour period.

METHODS

Methods common to all experiments

Male rats were used to avoid possible variations of iodine metabolism due to estrus cycles. The animals were divided into groups of eight or nine and the average body weights per group in any given experiment were approximately equal.

Since temperature and light variations have been shown to influence the diurnal regulation of metabolic activity in rats (Browman, 1937, 1943), constant environmental conditions were maintained. The temperature was 84° F. ± 4°, and the lights were turned off daily at 8 P.M. and on again at 8 A.M.

The diet of the animals varied. In Experiments I–IV, the animals received a low iodine diet supplying 1–2 μg. of iodine per 100 gm. animal per day (Remington diet 342 with 10% brewer's yeast). Before experimentation began the animals were kept on this diet for at least a month. This diet was known to produce some degree of activation of the thyroid gland with an increased uptake of radio-iodine (Leblond and Mann, 1942). The reason for using it was to reduce to a minimum the fluctuations in the amount of circulating iodide, which were found to be rather high in animals fed the iodine-rich commercial diets. For comparison, however, Experiment V was carried out on rats fed Purina Fox Chow, a diet supplying an adequate amount of iodine (5–10 μg. of iodine per 100 gm. animal per day).

The animals were given radio-iodine in all but Experiment I, which consisted simply of an estimation of the iodine content of the thyroids. In Experiments II–V, therefore, all animals received an injection of 25 μc. of radio-iodine, containing an amount of carrier iodine which varied somewhat from experiment to experiment. The animals were sacrificed exactly 4 hours after this injection, except in Experiment IV in which they were sacrificed at exactly 45 minutes. The times of the sacrifice were reported in the Tables as being 1 A.M., 5 A.M., 9 A.M., 1 P.M. and 5 P.M., with a possible variation up to plus or minus a half hour.

One thyroid lobe was homogenized in 50 cc. of 2N NaOH (except in Experiment V in which the two lobes were used). A 1 cc. aliquot was plated, dried in a 40° C. oven, and counted under a bell-shaped Geiger counter. The radioactivity content of the thyroid lobe examined was reported as percentage of the injected dose (Tables 2, 3 and 4). To facilitate comparison between the experiments the whole thyroid uptake was assessed by doubling the figures for the single lobe and was reported as such in Figures 1 and 2. The other thyroid lobe was fixed in Bouin for histological sectioning. Some of the sections were coated with medium lantern slide emulsion for localization of the radio-element by the radioautographic technique according to the method of Bélanger and Leblond (1946) as modified by Leblond *et al.* (1948).

The stomachs of the animals were weighed to ± 2 mg. in Experiments II–V. Since the rat stomach was shown to retain food for over 4 hours after a meal (Laugier and Leblond, 1943), the stomach weights gave an indication of the food eaten during the period of radio-iodine administration, and, when graphed, revealed the diurnal variations in food intake.

TABLE 1. IODINE CONTENT OF THE THYROIDS OF RATS FED A LOW IODINE DIET AND SACRIFICED AT 3 DIFFERENT TIMES OF DAY

(Experiment I)

Time of sacrifice	Iodine content of thyroid (μg.)
1 P.M.	0.39
5 P.M.	0.38
9 A.M.	0.50

Statistical analysis:
The Analysis of Variance showed no significant differences between the iodine content of the thyroids at these three different times of the day.

In all experiments, the data were statistically examined by means of analysis of variance. The probability value (P) was expressed as %, that is, the percentage of chances that the observed results are due to random error.

Methods special to each experiment

Experiment I. This experiment consisted of estimating the iodine content of the thyroid gland at three consecutive times, namely 1 P.M., 5 P.M. and the next morning at 9 A.M. Three groups of nine adult male rats on the low iodine diet were used. The body weights varied from 200–250 gm. The iodine content was estimated by the method of Chaney (1940) as modified in this laboratory (Table 1).

Experiment II. This experiment consisted of estimating the uptake of radio-iodine by the thyroid gland at six different times of the day in rats on the low iodine diet. The animals weighed from 104 to 116 gm., the average weights of the groups being 110 ± 3 gm. The 25 μc. of radio-iodine (supplied as carrier-free) were given by subcutaneous injection (Table 2, Figure 1.)

Experiment III. This experiment was similar to the previous one but, in addition to the thyroid uptake of radio-iodine, the amount of radioactivity present in the protein bound iodine of plasma was estimated, as well as the excretion of radio-iodine into stomach and urine.

The animals weighed from 150 to 200 gm., the average weights of the groups being 180 ± 7 gm. The dose of radio-iodide was mixed with 5 micrograms of non-radioactive

TABLE 2. RADIO-IODINE CONTENT OF THE LEFT THYROID LOBE OF RATS ON LOW IODINE DIET SACRIFICED AT 6 DIFFERENT TIMES OVER A 24 HOUR PERIOD AT 4 HOURS AFTER SUBCUTANEOUS INJECTION OF RADIO-IODINE

(Experiment II)

Number of group	Time of injection	Time of autopsy	Percentage of the injected dose of radio-iodine in the left thyroid lobe	Stomach weight (gms.)
1	1 P.M.	5 P.M.	4.9	0.91
2	5 P.M.	9 P.M.	5.0	2.67
3	9 P.M.	1 A.M.	4.8	2.11
4	1 A.M.	5 A.M.	4.0	3.24
5	5 A.M.	9 A.M.	6.0	2.05
6	9 A.M.	1 P.M.	6.6	1.17

Statistical analysis:
The Analysis of Variance shows no significant differences between the percentage of the injected dose of radio-iodine present in the thyroid at the various time intervals. The difference in stomach weights between any two groups is significant ($P < 5\%$) whenever it exceeds 1.08 gm.

iodide per animal before subcutaneous injection. The purpose of the addition of this carrier iodide was to raise the total inorganic plasma iodide to a sufficiently large level, so that the small amount of iodide entering the circulation from the food was negligible by comparison, i.e. the 5 micrograms would overcome the possibility of the incoming food iodide (1–2 μg. per day) significantly diluting the injected radio-iodide. Under these conditions, the specific activity of the plasma iodide available to the thyroid should be similar at all time intervals in spite of the variations due to food intake.

After sacrifice, the thyroid was examined as indicated.

The plasma was separated into an inorganic non-protein bound fraction (NPBI) and an organic protein bound fraction (PBI). The separation was done by precipitation with a double volume of 10% trichloracetic acid containing 20 μg. of iodide per cc. The mixture was shaken for 2 minutes and centrifuged. The clear supernatant was removed and

TABLE 3. RADIO-IODINE CONTENT OF THE RIGHT THYROID LOBE, STOMACH, URINE AND BLOOD FRACTIONS OF RATS ON LOW IODINE DIET SACRIFICED AT 6 DIFFERENT TIMES OVER A 24-HOUR PERIOD AT 4 HOURS AFTER SUBCUTANEOUS INJECTION OF RADIO-IODINE

(Experiment III)

Number of group	Time of injection	Time of autopsy	Percentage of the injected dose						Stomach weight (gms.)
			Right thyroid lobe	Stomach	Urine	Total P.B.I.	Total N.P.B.I.	Total plasma	
1	1 P.M.	5 P.M.	19.8	6.74	12.5	0.0676	1.66	1.73	1.65
2	5 P.M.	9 P.M.	17.7	7.57	28.1	0.0577	1.75	1.81	2.93
3	9 P.M.	1 A.M.	20.1	6.82	37.2	0.0820	1.04	1.12	3.23
4	1 A.M.	5 A.M.	18.6	6.67	35.4	0.0627	0.871	0.934	3.46
5	5 A.M.	9 A.M.	18.5	8.73	17.4	0.0700	1.64	1.71	3.23
6	9 A.M.	1 P.M.	18.9	6.56	14.5	0.0670	1.74	1.81	1.80

Statistical analysis:
The Analysis of Variance shows no significant differences between the percentage of the injected dose of radio-iodine present in the thyroid, stomach or total P.B.I. at the various time intervals. The difference in stomach weights between any two groups is significant ($P < 5\%$) whenever it exceeds 1.28 gm.

TABLE 4. RADIO-IODINE CONTENT OF THE RIGHT THYROID LOBE, STOMACH AND BLOOD FRACTIONS OF RATS ON LOW IODINE DIET SACRIFICED AT THREE DIFFERENT TIMES OVER A 24-HOUR PERIOD AT 45 MINUTES AFTER INTRAPERITONEAL INJECTION OF RADIO-IODINE

(Experiment IV)

Number of group	Time of injection	Time of autopsy	Percentage of the injected dose					Stomach weight (gm.)
			Right thyroid lobe	Stomach	Total P.B.I.	Total N.P.B.I.	Total plasma	
1	1 A.M.	1.45 A.M.	3.5	2.08	0.129	0.218	0.351	4.88
2	9 A.M.	9.45 A.M.	3.8	2.03	0.145	0.329	0.474	3.36
3	5 P.M.	5.45 P.M.	3.8	1.46	0.134	0.333	0.464	2.21

Statistical analysis:
The Analysis of Variance shows no significant differences between the percentage of the injected dose of radio-iodine present in the thyroid, stomach or total P.B.I. at the various time intervals. The difference in stomach weights between any two groups is significant ($P<5\%$) whenever it exceeds 1.40 gm.

the acid precipitate washed twice with an equal volume of 5% trichloracetic acid containing 10 μg. of iodide per cc. The precipitate was then dissolved in about 2–3 cc. of 0.5N NaOH. The material was again precipitated and washed with trichloracetic acid. The resulting precipitate was dissolved in 2 cc. of 0.5N NaOH solution, one cc. of which (PBI) was plated, dried in a 45° C. oven and counted in the Geiger counter. Similarly 1 cc. of combined supernatant and washings (NPBI) was plated, dried and counted.

The excretion of I^{131} into the urine and into the stomach were also examined. The urine was collected in large enamel trays containing 2N NaOH. One tray was placed under each cage containing three animals so that the I^{131} collected represented the total amount present in the urine of three animals. Each stomach, with its food contents, was weighed and homogenized in 2N NaOH, and a suitable aliquot was plated, dried and counted with the Geiger counter (Table 3, Figure 2).

TABLE 5. RADIO-IODINE CONTENT OF THE WHOLE THYROID, STOMACH, URINE AND BLOOD FRACTIONS OF RATS ON A NORMAL IODINE INTAKE AT 6 DIFFERENT TIMES OVER A 24-HOUR PERIOD AT 4 HOURS AFTER SUBCUTANEOUS INJECTION OF RADIO-IODINE

(Experiment V)

Number of group	Time of injection	Time of autopsy	Percentage of the injected dose						Stomach weight (gms.)
			Whole thyroid	Stomach	Urine	Total P.B.I.	Total N.P.B.I.	Total plasma	
1	9 A.M.	1 P.M.	6.2	15.9	25.4	0.1170	2.25	2.37	2.28
2	1 P.M.	5 P.M.	4.4	16.6	19.5	0.0958	2.82	2.92	2.35
3	5 P.M.	9 P.M.	5.5	11.0	32.5	0.0899	2.43	2.52	2.65
4	9 P.M.	1 A.M.	3.6	10.7	32.0	0.0705	2.12	2.19	3.00
5	1 A.M.	5 A.M.	3.8	11.9	48.0	0.0726	1.61	1.68	5.15
6	5 A.M.	9 A.M.	5.2	14.8	44.9	0.0716	2.11	2.18	4.26

Statistical analysis:
The Analysis of Variance shows no significant differences between the percentage of the injected dose of radio-iodine present in the thyroid at the various time intervals ($P>20\%$). The difference in the P.B.I. radioactivity between any two groups is significant ($P<5\%$) whenever it exceeds 0.0322. This is the case between the first group and any of the last three groups. The differences in stomach weights between any two groups is significant ($P<5\%$) whenever it exceeds 1.72 gm. The difference in the radioactivity of the stomach between any two groups is significant ($P<5\%$) whenever it exceeds 2.12.

Experiment IV. Since in Experiments II and III, the animals were sacrificed four hours after injection, the estimates of radioactivity in the thyroid and blood were only average indices of possibly varying rates of function within that time interval. The possibility of variations in activity within such intervals was examined in low iodine diet animals sacrificed at 45 minutes after injection. The animals weighed from 230 to 290 gm., the average weights of the groups being 248 ± 7 gm. Three periods spaced eight hours apart throughout 24 hours were investigated. Each animal received an intravenous injection of 25 μc. of I^{131} with 5 μg. of carrier iodide 45 minutes before sacrifice. The percentage of the injected dose present in the thyroid, stomach, PBI, NPBI, and total plasma were recorded, as well as the stomach weight in grams (Table 4).

Experiment V. In order to extend the results to animals fed various diets, an experiment identical to Experiment III was carried out in animals receiving the commercial diet Purina Fox Chow which includes an adequate amount of iodine. The animals weighed from 130 to 170 gm., the average weights of the groups being 250 ± 6 gm. The only other difference from Experiment III was that the amount of carrier iodide added to the radio-iodine injected was 20 μg.[1] (Table 5, Figure 2).

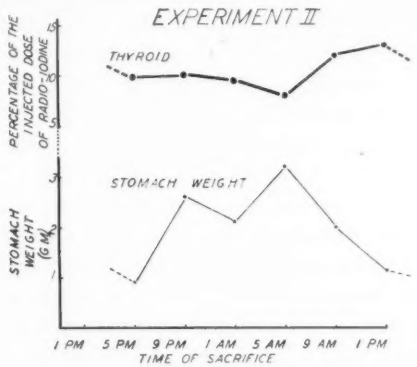

FIG. 1. Influence of the time of day on the percentage of the injected dose of radio-iodine taken up by the thyroid gland 4 hours after injection (Experiment III). The time indicated on the abscissae is the hour of sacrifice of the animals. The curve of the thyroid gland is shown as a heavy line. The stomach weights at various times of the day are plotted in the lower part of the figure.

RESULTS

Experiment I consisted of comparing the iodine content of the thyroid glands of rats on the low iodine diet sacrificed at three different times over a 20-hour period, namely 1 P.M., 5 P.M. and 9 A.M. The results (Table 1) showed no statistically significant variations in the iodine content of the

[1] It was thought that a larger amount of carrier was needed to buffer the higher blood iodide level existing after meals in these normal animals. The actual amount, 20 μg., was chosen arbitrarily. It was known from a pilot experiment that the addition of 20 μg. of carrier iodide to the radio-iodine decreased the radio-iodine uptake of the thyroid by 17% when the comparison was carried out at 1 P.M. and by 18%, at 1 A.M.

gland at these three times. The value at 9 A.M. was high, although not significantly so, on account of two thyroids with an unusually high iodine content. If these two glands were excluded, the average iodine content for the 7 remaining glands became 0.39 μg. per gland—a figure almost identical to the means at 1 P.M. and 5 P.M., namely, 0.39 and 0.38 μg. per gland. This result indicated that the iodine content of the thyroid remained constant over the 20-hour period. The next step was to examine whether or not the amount of iodine entering the gland also remained constant.

Experiment II was designed to compare the fixation of carrier free radio-iodine by the thyroids of 6 groups of low iodine diet rats sacrificed at various times during a 24-hour period. The results (Table 2, Figure 1) revealed that the uptake of radio-iodine by the thyroid gland remained fairly constant. In fact, the analysis of variance—presumably the best method to assess this type of data—showed no statistically significant difference between any two groups. Radio-autographs showed the presence of radio-activity in the colloid in all follicles of all the glands at the 6 times examined. On the other hand, the intensity of the radioautographic images was not identical at the various time intervals, the difference being especially clear between the thyroids of the animals sacrificed at 5 A.M. and those at 1 P.M. A straight "t" test was then carried out between these two groups; and, in contrast to the analysis of variance, indicated a significant difference ($P < 5\%$).

In interpreting these results, statistically significant changes were found in the stomach weights (Table 2), which on examination of Figure 1, seemed to vary inversely with the radio-iodine uptake of the thyroid. In other words, there was a tendency towards a decreased uptake at times of stomach repletion, when presumably there was absorption of the food iodide. The food iodide entering the circulation would dilute the radio-iodide and, therefore, although the total amount of iodine entering the gland might remain constant, the radioactive fraction of this iodine would be smaller than at other time intervals. The obvious way to avoid this difficulty was to add a sufficient dose of carrier iodide to the radio-iodide to render negligible the eventual influx of food iodide. This was done in Experiment III.

Experiment III consisted in comparing the fixation of carrier containing radio-iodine by the thyroids and, in addition, its release as PBI in 6 groups of rats on a low iodine intake sacrificed at various times during a 24-hour period. The results (Table 3; Figure 2, left) showed no statistically significant variations by analysis of variance "t" tests methods in either the radioactivity of the thyroid nor of the PBI. Therefore, the uptake of iodine by the gland and the amount of circulating iodine released from the gland remained constant throughout the 24-hour period.

These results may be contrasted with the other results of this same experiment which showed pronounced variations over 24 hours. The stomach weights (Table 3; last curve on the left of Figure 2) showed a maximum at 5 A.M. and fell sharply to a minimum between 1 P.M. and 5 P.M. This difference was highly significant ($P < 1\%$) and corresponded to that observed in the stomach weights of the previous experiment.

The radio-iodine excreted into the urine (Table 3; Figure 3, left) showed a marked rise between 9 P.M. and 5 A.M. to a maximum of 37.2% at 1 A.M. and a fall between 9 A.M. and 5 P.M. to a minimum of 12.5% at 5 P.M., a variation in good correlation with that of stomach weights. On the other hand, the amount of I^{131} released into the stomach remained steady throughout the entire period investigated, with only non-significant variations.

The NPBI of plasma (Table 3; Figure 2, left) showed a marked drop at 1 A.M. and 5 A.M. to a minimum at 5 A.M. of 0.87% of the injected dose. The values observed between 9 A.M. and 9 P.M. remained steady—the maximum of 1.75% being reached at 9 P.M. This curve was in inverse correlation with both the urine radioactivity and the stomach weights. It should be noted that the NPBI count was a figure indicating only the level of circulating iodide at the time of sacrifice, that is the net result of what happened to a highly dynamic system after four hours; and, therefore, this count was not an indicator of the amount of inorganic iodide available to the thyroid over the previous four hours.

The total plasma I^{131} observed (Table 3, Figure 2) followed a curve almost identical with that of the NPBI. This was to be expected since the relatively small and steady PBI produced very little change in the shape of the NPBI curve when added to it at each point to give the total plasma values.

Experiment IV was devised to show whether the amount of radio-iodine taken up by the thyroid would also remain the same at various times of the day, when the animals were sacrificed early after injection, that is, at 45 minutes instead of 4 hours as in previous experiments (Table 4). The results again showed no statistically significant differences between the three periods examined, whether the radio-iodine uptake of the thyroid or the radioactivity of the plasma PBI were considered.

The stomach weights (Table 4) showed a significant high of 4.88 gms. and low of 2.21 gms. at 1 A.M. and 5 P.M. respectively ($P < 1\%$), a variation in agreement with previous observations. The stomach counts varied from 1.46% at 5 P.M. to 2.08% at 1 A.M., the difference between these two groups being insignificant ($P > 10\%$).

The total NPBI count was significantly higher at 9 P.M. when it was 0.333%, than at 1 A.M., when it was 0.218 % of the injected dose. The counts

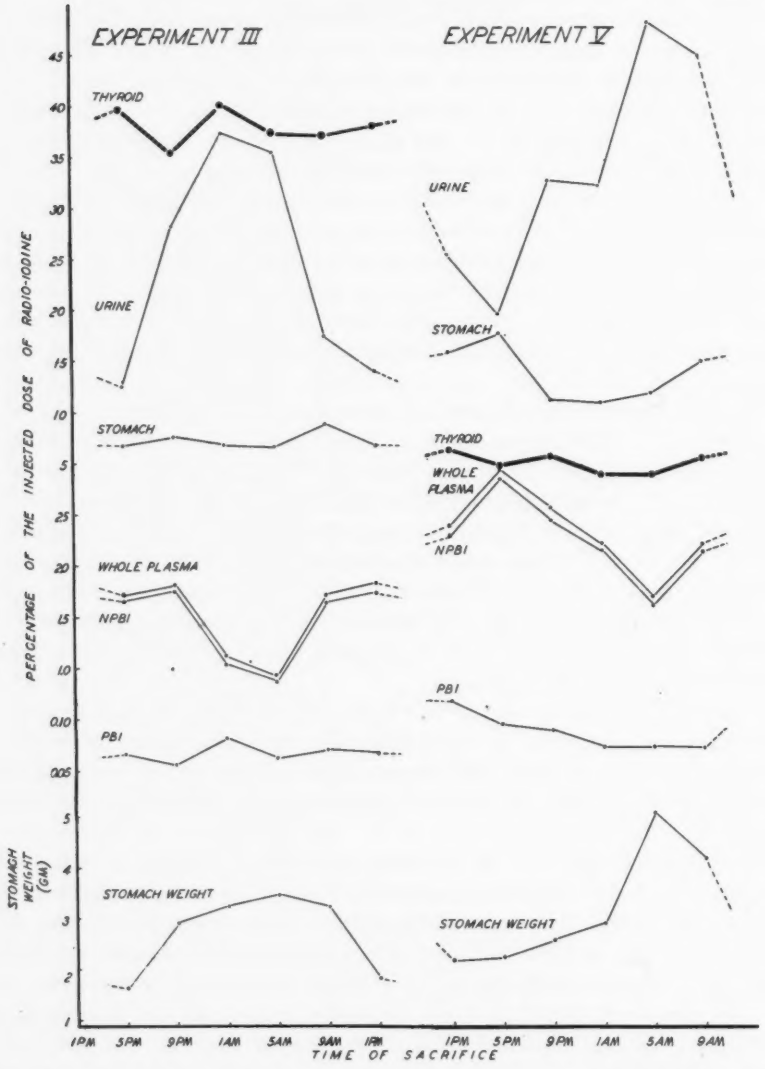

FIG. 2. Influence of the time of the day on the percentages of the injected dose of radio-iodine taken up by the thyroid gland 4 hours after injection in animals on a low iodine diet (Experiment III left hand side) and in animals receiving an adequate iodine intake (Experiment IV, right hand side). The time indicated on the abscissae is the time of sacrifice of the animals. The curves for the thyroid gland are shown as heavy lines. The percentages of the injected dose of radio-iodine present in urine, stomach, whole plasma, NPBI and PBI are also indicated. The stomach weights at the various times of the day are plotted in the lower part of the figure.

showed an inverse correlation with the stomach weights as was seen before.

Experiment V was designed to answer the question as to whether results similar to those of Experiment III could be obtained when using animals with a normal dietary iodine intake, in this case provided by a diet of Purina Fox Chow.

The radio-iodine uptake of the thyroid (Table 5; Figure 2, right) showed greater individual variations than in the previous experiments; but remained apparently constant throughout the day, since no statistically significant variations could be detected by analysis of variance or straight "t" tests. In contrast, the plasma PBI showed a significant variation in radioactivity content with a maximum at 1 P.M. and fall between 1 A.M. and 9 A.M. ($1\% > P > 5\%$). In this respect only the normal animals showed a difference from the iodine deficient animals of Experiments III and IV.

The radio-iodine excreted into the urine (Table 5; Figure 2) again showed a marked rise from a 5 P.M. minimum of 19.5% to a maximum of 48.0% at 5 A.M. The radio-iodine present in the stomach showed a rise at 5 P.M. to 16.6% and a fall to 10.7% at 1 A.M., this difference being significant ($P < 5\%$).

The NPBI radioactivity showed variations very similar to those of Experiment III (Figure 2). The maximum was reached at 5 P.M. and minimum at 5 A.M. The curve again showed an inverse correlation with both the urinary I^{131} and the stomach weights. The total plasma radioactivity followed that of the NPBI.

DISCUSSION

It was stated earlier that, if the thyroid activity is continuous, and the various phases of activity are performed simultaneously and at a constant rate, then the function of the gland may be described as involving a "steady state" of the thyroid metabolites. The most characteristic phases of thyroid activity are concerned with iodine metabolism. The handling of iodine by the gland may be sub-divided into at least five separate phases or functions, namely, the entry of inorganic iodide from the circulation, the synthesis of thyroglobulin from iodide in the cell apex, the deposition of thyroglobulin in colloid, the hydrolysis of thyroglobulin yielding thyroxine, and the passage of thyroxine into the circulation (Leblond and Gross, 1948, 1949). The thyroxine released into the circulation was found to be bound to proteins, thus forming the thyroid hormone or circulating protein bound iodine of the plasma (Gross *et al.*, 1950, 1951).

In this work only two of the phases of iodine metabolism in the thyroid were investigated, i.e., the rate of uptake of iodide from the circulating iodide, and the rate of secretion of thyroid hormone from the gland into the circulating PBI of the plasma, that is, the first and last phases of thyroid activity.

The rate of uptake of iodide by the thyroid was estimated by measuring the amount of radio-iodine found in the gland, four hours after injection. This represented the amount entering, minus that leaving the thyroid over the 4-hour period. However, the amount of radioactive PBI in the blood plasma was only about 0.2% of the amount of radio-iodine in the two thyroid lobes and, therefore the release of organically bound radio-iodine from the thyroid must have been small enough to make it possible to consider the thyroid count as a good index of the amount of radio-iodine entering the gland since the time of injection.

The problem of measuring the last phase of thyroid activity, i.e., that of hormone secretion from the gland, has been approached in this work by measuring the radioactivity present in the protein bound iodine of the circulating plasma. The figures obtained represented the net result of the total hormonal radio-iodine secreted from the gland since the time of injection, minus the total amount of radio-iodine lost from the plasma protein bound iodine by excretion, breakdown, and absorption into the body tissues. If it is assumed that the rate of loss of hormone from the plasma is fairly constant, then the PBI counts depended directly on the average rate of radio-hormone output by the thyroid gland since the time of injection.

Three points had to be proved to demonstrate the existence of a "steady state" of the thyroidal iodine. The first problem was whether the thyroid activity was continuous or intermittent. When this problem was examined the thyroid gland was found to be taking up some iodine in all of the four hour periods examined, from whatever time of day these periods were selected (Tables 2, 3, and 4). This was true also of the 45-minute periods examined (Table 5). That some secretion also took place at all times was indicated by the presence of labeled PBI in the plasma of all groups (Tables 3, 4, and 5). Thus, there was no interruption in the activity of the thyroid throughout the day either in iodine deficient or in normal animals.

The second question, which asked whether the various phases of thyroid activity are carried out singly or simultaneously, was answered by the same data which also showed that both the radio-iodine uptake by the thyroid and the formation of radio-PBI took place concurrently (Tables 3, 4, and 5). In addition, examination of individual data indicated that the PBI radioactivity was high when the thyroid radio activity was high and vice versa. This impression was tested by a statistical analysis of the correlation between the figures for thyroid and PBI. This was carried out in Experiment III. The correlation between thyroid and PBI radioactivity in this experiment was equal to 0.33 and a "t" test of its significance revealed it to be a true correlation ($P = 2\%$). This result further demonstrated a close relation between the two thyroid activities.

The third problem, namely, whether the thyroid activity occurred at a constant or at a varying rate, was not easily solved. In Experiment II (Table 2), the thyroid count was found to be fluctuating slightly over 24 hours. If the amount of iodine entering the gland had remained constant, while, at times of feeding, the amount of circulating iodine had been raised by the absorption of the food iodide, then the tracer dose of radio-iodide given at such times would be diluted in the circulation and less of it would enter the gland. This cause of error was eliminated by adding to the radio-iodide a small amount of carrier (5 to 20 μg.), which was sufficient to make the food iodide negligible by comparison. Thus, at all times, the radio iodine would have the same dilution in the circulation. Unfortunately, this advantage was gained at the expense of the more physiological condition obtained with carrier free radio-iodine as in Experiment II. However, the Experiments III–V done with carrier demonstrated at least that under these conditions (Tables 3, 4, and 5) there was a constant rate of iodine fixation by the thyroid. The constancy is all the more remarkable, when compared to the considerable variations in other phases of iodine metabolism revealed by the urinary excretion and the NPBI (Figure 2). At any rate, if any fluctuations in uptake of iodine existed, they were not detectable and must be very small indeed. The suspicion that very small fluctuations may exist was supported by the PBI results. In two of three experiments no significant differences were observed between the radioactivity of the PBI at the various times of the day, but Experiment V showed a significant fluctuation of the PBI values. This variation was not necessarily due to a change in thyroid activity, since it might be accounted for by a modification in the excretion of the PBI. At any rate, this fluctuation was, however, so minute that, for most practical and theoretical purposes, it may be considered that the thyroid activity was performed at a constant rate. It was concluded that the results of Experiments I and II completed by III–V demonstrated that the requirements for the justification of the assumption of a "steady state" over a 24-hour period have been fulfilled, at least with respect to the first and last phases of thyroid activity.

With regard to the other phases, the radioautographs showed the presence of radioactivity in the colloid at 4 hours after injection whatever the time of the day. Therefore, in all cases, thyroglobulin had been secreted into the colloid. At any rate, thyroglobulin entry into the colloid and the other thyroid functions may be considered as part of a system in which five interdependent phases of activity are being carried out; then, if the first and last of these phases are shown to occur at a similar and constant rate, it must necessarily be concluded that the intermediate phases are being carried out at the same (net or average) rate.

Therefore, since the "steady state" of the thyroidal iodine has been

demonstrated, previously published arguments based on such an assumption may now be considered as being correct. Thus, the interpretation of radioautographic pictures on the assumption of a "steady state"(Leblond and Gross, 1948) had led to the conclusion that the function of the thyroid gland consisted of a series of activities in which all cells of all follicles were continuously involved and which proceeded at a definite characteristic rate for each thyroid follicle. These activities are the extraction of iodide from plasma, the synthesis of thyroglobulin in the apex of the follicular cell, the deposition of thyroglobulin into the colloid, the proteolysis of thyroglobulin to its amino-acid components in the colloid, and the passage of thyroxine through the cell into the plasma. Since a steady state of thyroidal iodine is demonstrated by the present work that all thyroid activities are proceeding continuously, simultaneously and at a constant rate throughout a 24-hour period.

However, a comparison of the three experiments in which the animals were sacrificed 4 hours after injection showed that the grand averages of the thyroid uptakes of radio-iodine for each experiment were very different. Thus, the thyroids of the small rats used in Experiment II took up less iodine than those of the larger rats used in Experiment III. Therefore, the level at which a "steady state" is established may possibly change with the age of the animals. Similarly, the thyroids of the Purina fed rats of Experiment V took up less radio iodine than the glands of the rats fed the low iodine diet. Since the latter had been raised on a Purina diet before being given the low iodine diet, here again the level of the "steady state" had changed following the diet change. Temperature and other factors may also alter the level of "steady state." Presumably, the rate of thyrotrophin secretion by the hypophysis plays a major role in the establishment of the rate of thyroid activity.

Briefly, it appears that, over relatively short periods of time, the rate of the various functions of the thyroid gland is remarkably steady. But the level of activity is not rigidly fixed and may be gradually modified by many factors, so that, under the changed conditions, a new "steady state" will be established.

SUMMARY

The problem of the existence of a "steady state" of the thyroidal iodine was examined by comparing the behavior of this iodine at various time intervals over 24-hour periods. Two types of experiments were set up using male rats kept under controlled conditions of environment and fed a low iodine diet namely: 1) the iodine content of the thyroid was examined at 3 different times during a 24-hour period; 2) the radio-iodine uptake of the thyroid and the radioactivity of the protein bound iodine of plasma were

measured at 6 different times of the day in animals sacrificed 4 hours after radio-iodine injection. This experiment was repeated under various conditions, including the use of animals fed a diet with a normal iodine intake.

The results demonstrate that: 1) the iodine content of the thyroid shows no diurnal variation (Table 1); 2) the uptake of iodine and the release of iodine containing hormone occur *simultaneously, continuously* and at an approximately *constant rate* (Tables 2–5, Figs. 1 and 2). That a deviation from the constancy of the rate of thyroid secretion may occur was indicated by a significant, but small variation in the radioactivity of the protein bound iodine of plasma in one of three experiments (Table 5). Thus, possibly, minor fluctuations in the rate of thyroid functions may occur, but, if they exist in all cases, they are generally too small to be detected.

As an approximation satisfactory for most practical and theoretical studies of the thyroid gland, it is concluded that the rates of iodine entry and hormonal iodine output are constant. These rates must also be equal to each other since the iodine content of the gland remains constant. Therefore, the thyroidal iodine is in a "steady state."

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THE PRESENCE OF FREE IODINATED COMPOUNDS IN THE THYROID AND THEIR PASSAGE INTO THE CIRCULATION

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WHILE it is generally believed that the iodine of the thyroid is either bound in the thyroglobulin molecule or free as the iodide ion, recent evidence has indicated that other iodine containing substances are present in the gland. Thus, free thyroxine has been detected in extracts of unhydrolyzed thyroids (Leblond and Gross, 1949; Gross *et al.*, 1950); free diiodotyrosine, monoiodotyrosine and several other, as yet unidentified, iodine compounds have also been found in the gland (Tishkoff *et al.*, 1949; Gross *et al.*, 1950). The thyroglobulin molecule contains these amino-acids bound in peptide linkage, as shown by Harington for thyroxine and diiodotyrosine (1933) and by Fink and Fink for monoiodotyrosine (1948).[1] Therefore, it has been presumed that these free amino-acids are derived from thyroglobulin through the action of a proteolytic enzyme system such as the one demonstrated by de Robertis in the thyroid (1949). Furthermore, this pool of amino-acids appears to be the source of the plasma thyrozine, the presumed thyroid hormone (Leblond and Gross, 1949; Gross *et al.*, 1950). To elaborate these findings, the relative amounts of labeled iodinated amino-acids in thyroid and plasma has been investigated at various time intervals after the administration of radioactive iodide.

METHODS

Female albino rats weighing 170–200 grams were injected with 100 microcuries of radioactive iodide subcutaneously and sacrificed at various intervals thereafter. Three groups of animals were used, namely:

a) Animals fed a commercial diet, Purina Fox Chow, which supplied a more than adequate iodine intake (approximately 10 μg. daily in animals of this weight);
b) Animals fed a diet low in iodine (Remington No. 342 (1937), with 10% yeast) which supplied approximately 2 μg. daily in animals of this weight;
c) Animals fed the same low iodine diet plus 20 μg. of iodide daily given by subcutaneous injection in two doses, by which means a moderately high iodine intake was achieved.

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[1] The results of Fink *et al.* (1947, 1948) were recently confirmed by Tishkoff *et al.* (1949), Taurog *et al.* (1950) and Roche *et al.* (1950.

TABLE 1. EXPERIMENTAL DETAILS

Time after injection of I^{131}	Number of animals used		
	Adequate iodine intake (Purina)	Low iodine intake (Remington No. 342 +yeast)	Moderately high iodine intake (Remington No. 342 +yeast +20 μg. I^- daily)
15 mins.	3	2	
1 hr.	4	4	2
3 hrs.	4	4	2
24 hrs.	4	2	2
48 hrs.	4	2	2

The number of animals on each iodine intake for each time interval investigated is recorded in Table 1.

All animals were anaesthetized with ether and then as much blood as possible was drawn from the descending aorta with a heparinized syringe. The thyroids were rapidly removed and ground up in 0.5 cc. of cold saline in a glass mortar. The gland homogenates and the blood plasmas were first extracted with twice their volume of water-saturated n-butanol. Three further extractions were carried out with an equal volume of solvent. The radioactivity of the combined butanol extracts and butanol-insoluble residues was estimated by means of a Geiger-Muller counter. Addition of the two counts gave the amount of radio-iodine in the gland. This was expressed as percentage of the injected dose (Table 2, columns 2, 4 and 6), while the radio-activity of the butanol extract was expressed as percentage of the thyroidal radioactivity (Table 2, columns 3, 5 and 7).

The combined butanol extracts were then reduced to about 0.1 cc. at room tempera-

TABLE 2. AMOUNTS OF RADIOIODINE FOUND IN THE BUTANOL EXTRACT OF UNHYDROLYZED THYROID AND IN THE WHOLE GLAND AT VARIOUS TIMES AFTER THE SUBCUTANEOUS INJECTION OF NaI^{131} IN RATS FED DIETS WITH DIFFERENT IODINE LEVELS

Time after injection		Adequate iodine (Purina)		Low iodine		Moderately high iodine (Remington 342 +yeast +20 μg. I^- daily)	
		% injected dose in whole thyroid	% thyroid I^{131} in butanol extract	% injected dose in whole thyroid	% thyroid I^{131} in butanol extract	% injected dose in whole thyroid	% thyroid I^{131} in butanol extract
15 mins.	a	0.6	9.4	6.6	0.9		
	b	0.3	7.8	4.5	1.0		
	c	0.3	11.2				
1 hr.	a	1.2	3.2	17.9	0.9	1.3	1.8
	b	2.5	4.3	17.6	2.7	2.3	1.6
3 hrs.	a	4.5	1.9			1.2	2.0
	b	2.8	1.9			1.3	1.7
24 hrs.	a			31.8	0.9	6.9	0.6
	b	7.2	1.3	14.0	2.3	7.0	0.4
48 hrs.	a	5.3	1.8	7.6	3.6	1.0	1.0
	b	5.3	1.5	17.1	2.0	2.3	1.2
	c	11.2	2.0				
	d	12.6	0.7				

ture *in vacuo*. 0.02 cc. aliquots were placed on filter paper for chromatography by a modification of the method of Fink *et al.* (1947, 1948) as previously described (Gross *et al.*, 1950). The solvent used in the first dimension consisted of n-butanol saturated with 2N formic acid, and, in the second dimension, of a mixture of 4 parts of n-butanol and 1 part of dioxane saturated with 2N ammonium hydroxide. The chromatograms of the animals fed an adequate iodine intake were done in this manner, but in the chromatograms of the animals fed the low iodine diet with or without iodide supplement, the first dimension solvent was changed to butanol saturated with phosphate buffer at pH 7.2. This change was made as it had been found that in the acid medium of the butanol-formic mixture, iodide tended to oxidize to iodine, which has a much greater Rf; and this caused an elongation and upward displacement of the iodide spot. The other iodine compounds showed approximately the same relative location with both solvents.

For each sample, duplicate paper chromatograms were made with and without the addition of 100 μg. each of the following carriers: monoiodotyrosine, diiodotyrosine, thyroxine and diiodothyronine. After chromatography the papers were dried at room temperature and exposed to X-ray films for detection of radioactive spots by radio-autography as previously described (Gross *et al.*, 1950) all the films of the Purina fed animals were exposed for 20 days and developed at the same time. Similarly, the films of the animals on the low iodine diet were exposed for 5 days and developed at the same time.

The paper chromatograms embodying carriers were then subjected to the following procedure. A volume of cold sulphanilic acid (0.05M in 9% HCl) was added to an equal volume of cold 4.5% sodium nitrite and allowed to diazotize for 10 min. at 4° C. The paper was sprayed with this solution and left for about 5 minutes to allow evaporation of excess moisture. The damp paper was then sprayed with 5% Na_2CO_3 in 50% ethanol. Under these conditions, the thyroxine and diiodothyronine spots, due to the added carriers, reacted by producing a deep purple color, while the mono- and diiodotyrosine spots gave a more orange hue. The paper chromatograms were then compared to the corresponding X-ray films. Whenever a colored spot exactly coincided with a black spot on the X-ray film, it was concluded that the substance responsible for the colored spot was also present in a radioactive form. In the case of radio-autographs of chromatograms prepared without carriers, there were no significant differences in the location of the spots, which therefore were identified by comparison with the duplicate made from the chromatograms containing the carriers. All the figures in this paper are radio-autographs of chromatograms made without carriers and labeled by comparison.

In some cases, elemental iodine was used as a carrier; and its location was determined by spraying the chromatogram with a starch solution after the first dimension or with 1% palladium chloride after the second dimension run.

RESULTS

The radio-autographs of chromatograms from thyroid and plasma revealed a characteristic sequence in the animals receiving an *adequate iodine intake* from the commercial diet. At 15 minutes after radio-iodine injection the butanol extracts of thyroid and plasma showed only the presence of iodide on the radio-autographs. There were traces of mono- and diiodotyrosine in one animal. At 1 hour, the thyroids showed definite spots due to mono- and diiodotyrosine as well as iodide and a weak spot due to thyroxine, which, although distinct, was too faint to appear on the

photograph (Fig. 1). On the other hand, the plasmas (Fig. 2) contained only iodide. At 3 hours (Fig. 3) the intensity of all components in the thyroid extracts had increased and more definite amounts of thyroxine were present. The plasmas, in addition to the intense iodide reaction (Fig. 4), showed a very faint thyroxine spot not demonstrable on photographs. At 24 hours (Fig. 5) there was a relative decrease in the intensities of the monoiodotyrosine, diiodotyrosine and iodide constituents of the thyroid extract, with a pronounced increase in the radioactivity of the thyroxine spot. The plasma at this time (Fig. 6) showed an intense thyroxine spot with a much fainter iodide area. At no time interval were diiodotyrosine or monoiodotyrosine demonstrable in the plasma.

In addition to the identifiable spots described so far, weak spots due to unknown compounds were also observed. Thus, in the thyroids of one 3 hour and one 24 hour animal an unknown iodinated compound could be seen to the right of thyroxine as a barely visible spot corresponding in location to the unknown previously described as number 1 (Gross *et al.*, 1950; see also Fig. 7–12). Traces of the same substance were present in one of the 24-hour plasmas. Furthermore, a more distinct spot, previously described as unknown number 3, was recognized between the diiodotyrosine spot and the top of the iodide spot. This was found in all 3-hour and 24-hour animals and sometimes consisted of 2 or 3 rather than one distinguishable spot. The upper edge of the paper also showed the presence of some radioactivity with an occasional concentration near the upper right corner previously referred to as compound number 2 (Gross *et al.*, 1950). Finally, diiodothyronine was never found in thyroid nor plasma.

In the animals on a *low iodine intake*, monoiodotyrosine and to a lesser extent, diiodotyrosine could be demonstrated in the thyroid butanol extract as early as 15 minutes after injection. At 1 hour (Fig. 7) in addition to these two amino-acids and iodide, there was a strongly radioactive accumulation of thyroxine accompanied by intense spots for the two unidentified iodo-compounds, numbers 1 and 3. At 3 hours (Fig. 9) there was an increase in the radioactivity of all compounds except iodide, which showed a diminished intensity. At 24 hours (Fig. 11) the radioactivity in all spots had decreased except possibly that of compound number 1. The spot labeled number 5, present on the upper margin of the paper directly over iodide at 1 and 3 hours (Figs. 7 and 9), was identified as elemental iodine. The plasma preparations showed thyroxine, unknown number 1 and iodide at 1 hour after injection (Fig. 8), with a rapid diminution in the radioactivity of iodide and possibly unknown 1 with time (Figs. 10–12). At no time interval examined were any other iodinated compounds demonstrable in the butanol extract of plasma. The previously reported faint diiodotyrosine spot (Gross *et al.*, 1950) was due to exchange between radio-

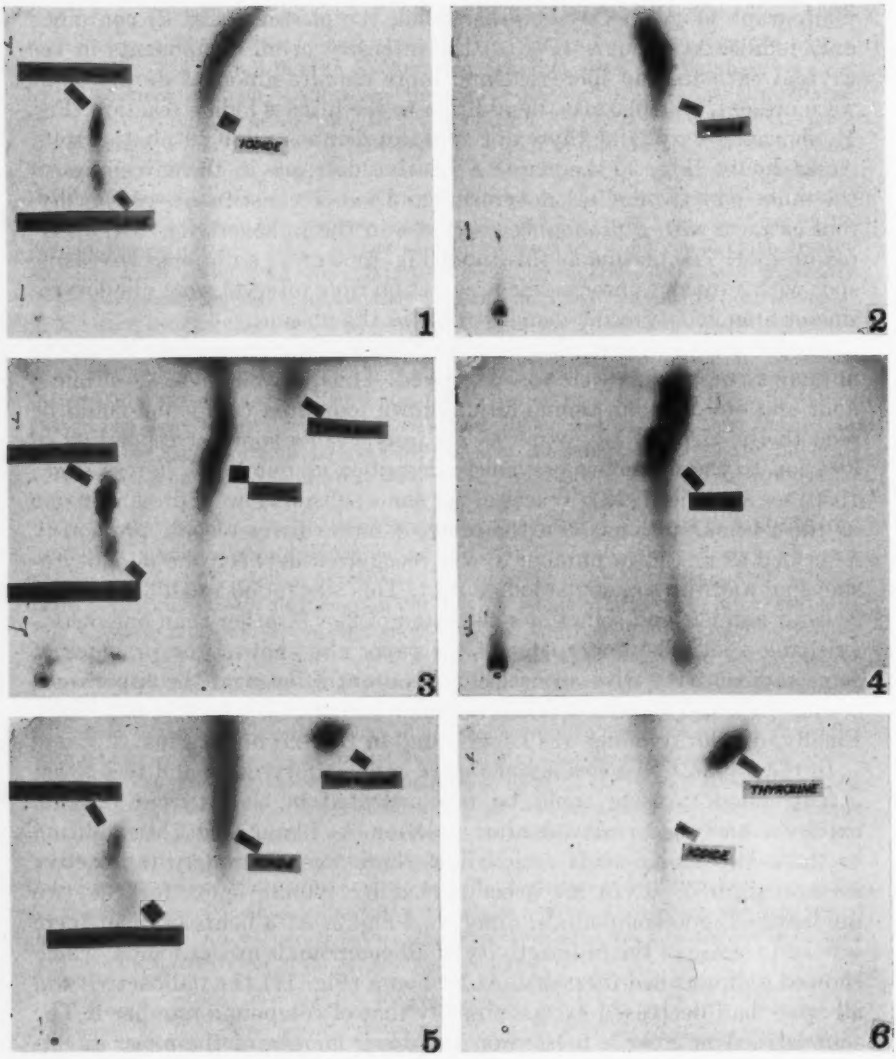

PLATE I. Radioautographs of paper chromatograms of the butanol extracts of unhydrolyzed thyroids (Figs. 1, 3 and 5) and plasmas (Figs. 2, 4 and 6) of rats receiving an adequate iodine intake (Purina Fox Chow). The animals received a subcutaneous injection of 100 microcuries of carrier-free radio-iodine. All radioautographs were given the same exposure.

iodide and the iodine of the carrier diiodotyrosine present on the paper. No traces of diiodotyrosine were found in the plasma of any animals when the chromatograms were prepared without carriers.

The chromatographic patterns obtained from the thyroids and plasmas of the animals on a *low iodine intake, receiving 20 micrograms of iodide per day,* were similar to those shown in Figs. 1–6 except that there was a slower rate of entry of radioactivity into the various amino-acids, radio-thyroxine appearing only at 24 hours.

The figures on the percent of the dose of radio-iodine taken up by the thyroid at various times after injection (Table 2) showed that iodine uptake is most rapid in the low iodine animals and slowest in the animals receiving the daily injection of 20 μg. of iodide. The figures may be taken as giving a rough indication of the rates of radio-thyroglobulin formation (Leblond and Gross, 1948). The proportion of thyroidal radioactivity present in the butanol extract was at first higher in the animals on the adequate iodine intake. Later, the levels became more or less similar in the three groups, although at 24 to 48 hours they may perhaps be greater in the iodine deficient group (mean 2.2) than in the Purina fed group (mean 1.5) and in the latter, greater than in the iodide injected group (mean 0.8).

DISCUSSION

Radio-autographs of thyroid sections had previously revealed that the iodine entering the thyroid gland of the rat is transformed into thyroglobulin in the apex of the thyroid cell and deposited as such in the colloid of the thyroid follicle (Leblond and Gross, 1948). The iodine entry was shown to be continuous throughout the 24 hours of the day; and so was the deposition of thyroglobulin in the colloid (Dougherty *et al.*, 1951). The "steady

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Explanation of Figures

Fig. 1. Thyroid extract of animal sacrificed 1 hour after radio-iodide injection. Besides the spots indicated on the figure, a thyroxine spot too faint for photography could be identified.

Fig. 2. Plasma extract of animal sacrificed 1 hour after radio-iodine injection. Iodide is the only definite spot. No trace of thyroxine could be seen.

Fig. 3. Thyroid extract of animal sacrificed 3 hours after radio-iodine injection. A clearcut thyroxine spot has appeared.

Fig. 4. Plasma extract of animal sacrificed 3 hours after radio-iodine injection. Besides iodide, a thyroxine spot too faint for photography could be identified.

Fig. 5. Thyroid extract of animal sacrificed 24 hours after radio-iodine injection. The thyroxine spot is now fairly intense.

Fig. 6. Plasma extract of animal sacrificed 24 hours after radio-iodine injection. Besides iodide a clearcut, intense, thyroxine spot has appeared.

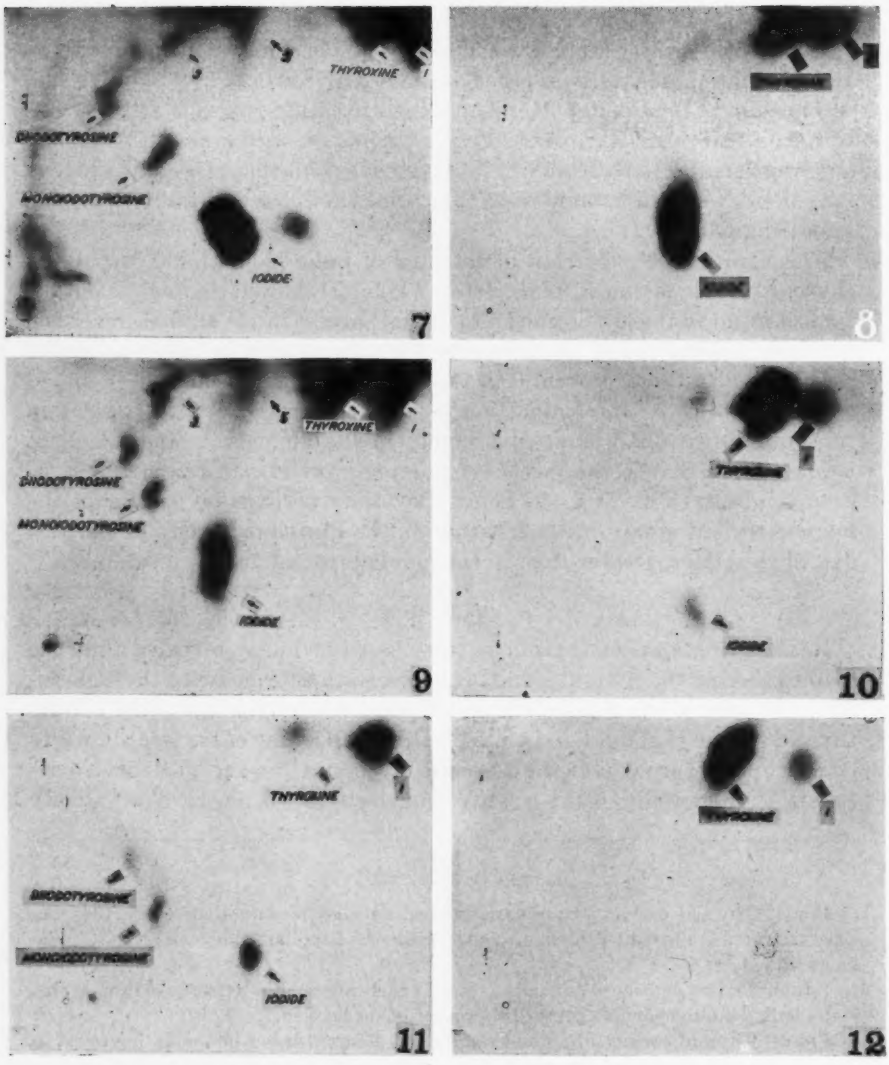

PLATE II. Radioautographs of paper chromatograms of the butanol extracts of unhydrolyzed thyroids (Figs. 7, 9 and 11) and plasmas (Figs. 8, 10 and 12) of rats receiving a low iodine diet. The animals received a subcutaneous injection of 100 microcuries of carrier-free radio-iodide. All radioautographs were given the same exposure.

state" required that an output of iodine balance the iodine entry; and therefore, the iodine bound as thyroglobulin in the colloid must change to a form capable of leaving the thyroid follicle and passing into the circulation.

Studies of thyroid glands by the method of isotope dilution revealed the presence of thyroxine not bound in peptide linkage (Leblond and Gross, 1949). This finding suggested that thyroxine came from the breakdown of thyroglobulin in the colloid of the thyroid follicle. Further, the free thyroxine of the thyroid would appear to be the source of the free thyroxine of plasma (Leblond and Gross, 1949).

However, the method of isotope dilution does not separate thyroxine from substances with very similar solubilities; and, therefore, an attempt was made to confirm these results by the method of paper chromatography. Preliminary observations on the thyroid gland confirmed the presence of free thyroxine and revealed that of monoiodotyrosine, diiodotyrosine, and several unknown iodo-compounds, all of which were readily extracted with butanol at room temperature in the absence of hydrolysis, and, therefore, were not bound in peptide linkage. Similarly, free thyroxine was found in plasma (Gross *et al.*, 1950).

The present work extended these results in two ways. Firstly, the published observations had been chiefly obtained with the highly stimulated thyroids of iodine deficient rats. It was possible here to make identical observations in animals fed a diet providing adequate iodine intake. Not only were free mono- and diiodotyrosine found in the thyroid, free thyroxine found in the thyroid and plasma (Figs. 1–6), but also the unknown substances, numbers 1 and 3 were observed in very small amounts in the

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EXPLANATION OF FIGURES

FIG. 7. Thyroid extract of animal sacrificed 1 hour after radio-iodide injection. Besides an intense iodide spot, it is possible to see intense spots for thyroxine and compound No. 1, as well as spots for monoiodotyrosine, diiodotyrosine, compounds Nos. 3 and 5.

FIG. 8. Plasma extract of animal sacrificed 1 hour after radio-iodide injection. Besides iodide, only thyroxine and compound No. 1 are visible.

FIG. 9. Thyroid extract of animal sacrificed 3 hours after radio-iodide injection. There are no clearcut changes in the pattern except for a decrease in the iodide spot.

FIG. 10. Plasma extract of animal sacrificed 3 hours after radio-iodide injection. There is a decrease in the iodide spot.

FIG. 11. Thyroid extract of animal sacrificed 24 hours after radio-iodide injection. The thyroxine spot shows a decreased intensity.

FIG. 12. Plasma extract of animal sacrificed 24 hours after radio-iodide injection. The thyroxine spot is still intense.

gland. Therefore, the presence of small quantities of these various iodo-compounds in the thyroid was truly physiological.[2]

The second result of this series of experiments was the establishment of a sequence of events in the thyroid glands in agreement with basic theory. This sequence of events will be described in detail for the series of *animals on an adequate iodine intake*. At 15 minutes after injection, radio-iodide was the only substance detected by chromatography in the butanol extracts of plasma and thyroid in 2 out of 3 animals. The circulating radio-iodide had, therefore, entered the thyroid gland. Furthermore, the results in Table 2 showed that the butanol extractable radioactivity—in this case, iodide—made up 7.8 to 11.2% of the total radioactivity of the gland. The balance must have consisted mainly of radio-thyroglobulin, the only butanol insoluble iodo-compound known to exist in the thyroid. Therefore, at 15 minutes, thyroglobulin had been synthesized in appreciable amounts from the injected radio-iodine. The iodination leading to the formation of thyroglobulin may be due to the formation of elemental iodine from iodide (Harington and Pitt-Rivers, 1945). The identification of spot number 5 as elemental iodine in a number of chromatograms (Figs. 7 and 9) supported this hypothesis. However, the possibility that this elemental iodine was an artefact of the manipulation has not been satisfactorily eliminated.

At 1 hour after injection, radioactive spots due to iodide, monoiodotyrosine, diiodotyrosine (Fig. 1) and traces of thyroxine were detected in the thyroid gland extracts. At 3 hours, the thyroxine spot of the thyroid extracts was more definite (Fig. 3) and the unknown substances number 1 and 3 had appeared. The biological source of these iodo-compounds must have been the thyroglobulin formed earlier from radio-iodide, since thyroglobulin was known to contain thyroxine and diiodotyrosine (Harington, 1933), as well as monoiodotyrosine and unknown iodinated substances (Fink *et al.*, 1947, 1948). An enzyme hydrolyzing this protein to its component amino-acids would satisfactorily account for the production of these small molecules. The enzyme of de Robertis (1949), which was first identified in the colloid (where thyroglobulin is also located) and was later estimated by its ability to release tyrosine from a protein substrate, may

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[2] The possibility of the results being due to the radio-chemical effect of radio-iodine were examined in a previous report (Gross *et al.*, 1950). It was possible to show that free monoiodotyrosine, diiodotyrosine and thyroxine could be identified after injection of 1 μc. of radio-iodine to rats on a low iodine diet. This dose of radioactivity was minute enough to eliminate the possibility that the presence of these three substances was due to a radio-chemical effect. However, unknowns 1, 3 and 5 were not visible with this low dose and might thus be radio-chemical artefacts. This possibility was considered unlikely because of the early appearance of the unknowns (at 3 hours in the Purina-fed animals and 1 hour in the low iodine animals).

be the agent responsible for the liberation from thyroglobulin of the iodinated compounds detected in the thyroid.

Most of these compounds must be metabolized within the gland, since neither diiodotyrosine, nor monoiodotyrosine, nor compound(s) 3 were seen in the plasma extracts of any of the animals. The presence of diiodotyrosine in blood plasma was claimed by several authors (Salter, 1941; Morton *et al.*, 1941), but methods of low specificity had been used. The present results would indicate that diiodotyrosine was not released by the thyroid gland into the circulation. The fate of this substance within the thyroid—as well as that of monoiodotyrosine and several other unknown compounds—may be to undergo dehalogenation with release of iodide to the iodide pool of the gland. An alternative possibility is a reincorporation of these amino-acids as such in the thyroglobulin molecule.

At 1 hour after injection of radio-iodide to the animals fed a diet adequate in iodine, nothing but radio-iodide could be detected in the blood (Fig. 2), while a small amount of thyroxine was present in the thyroid. As time elapsed and the amount of labeled thyroxine in the thyroid increased (Figs. 3 and 5), this substance appeared in the plasma, where traces of it were recognizable at 3 hours and significant amounts were found at 24 hours (Fig. 6). The hypothesis that free radiothyroxine is formed in the thyroid before passing into the plasma required that soon after administration of radio-iodine, the amount of radioactivity per unit weight—or specific activity—be greater in the thyroid thyroxine than in the plasma thyroxine. This conclusion may indeed be drawn from the present results for the following reason. Recent, unpublished iodine determinations revealed that the intensity of the thyroxine spots of the films happened to be a fair index of the specific activity of this substance. Therefore, it appears correct to conclude from the results in plate 1 that, following radio-iodine injection, radio-thyroxine appears in the thyroid gland, whence it moves to the circulation.

The presence of both thyroglobulin and de Robertis' proteolytic enzyme in the colloid of the thyroid follicles implied that thyroxine was formed from the interaction of these two substances in the colloid, and, therefore, entered the circulation by passage throuh the cytoplasm of the cells. This passage was favored by a gradient of thyroxine concentration between gland and plasma. If the results of the unpublished iodine determinations quoted above were recalculated as concentrations, it could be seen that the concentration of free thyroxine was more that 100 times higher in thyroid than in plasma.

The sequence of events was less distinct, but, as far as could be ascertained, identical in the *animals on a low iodine intake*. There was a higher uptake of radio-iodine by the thyroid (Table 2) and a more rapid appear-

ance of free thyroxine in thyroid and plasma. The only apparent difference in this series was the presence in the plasma of a large amount of compound number 1. This substance may be secreted by the thyroid gland in a manner similar to thyroxine. An alternative possibility was that compound number 1 was a degradation product of thyroxine resulting from the metabolism of this substance within or outside the gland. Unpublished results showing the presence of this substance in the plasma after radio-thyroxine injection into thyroidectomized animals favor the latter view. At any rate, iodide, thyroxine and compound number 1 made up the radioactivity of the butanol extract of plasma.

In the *animals on a moderately high iodine intake,* there was a smaller uptake of radio-iodine (Table 2) and a less rapid appearance of free thyroxine in thyroid and plasma than in the other groups, but the sequence of events seemed to be unchanged.

In conclusion, the function of the thyroid gland may be summarized as follows. Circulating iodide is collected by the thyroid cells and promptly transformed into thyroglobulin. Previous results had shown that thyroglobulin was formed in the cell and later transferred to the colloid of the thyroid follicle. An enzymatic system present in the colloid hydrolyzes thyroglobulin to its component amino-acids, thus resulting in the release of monoiodotyrosine, diiodotyrosine and thyroxine, as well as several unidentified substances. Some time after thyroxine can be detected in the thyroid, this substance appears in the circulation. A gradient of concentration of over 100:1 between the concentration of free thyroxine in the thyroid and that in the plasma may play a role in the passage of this substance into the circulation.

SUMMARY

The formation of iodinated substances in the thyroid gland and their eventual passage into the circulation was examined in rats receiving low, adequate or moderately high iodine intakes. The animals were given radio-iodine and sacrificed at 15 minutes, 1, 3, 24, or 48 hours. Butanol extracts of their thyroids and plasma were chromatographed on paper. Radio-autographs of the papers were then obtained, thus making it possible to detect the labeled substances formed subsequent to the injection of radio-iodine.

The thyroid extracts contain iodide, monoiodotyrosine, diiodotyrosine, thyroxine and at least two unidentified iodine-containing substances described as compounds number 1 and 3. Another compound (number 5) may be identified as elemental iodine, but its presence as such in the thyroid gland is not yet definitely proved by the available data.

The plasma extracts contain only iodide, thyroxine and, at least in iodine deficient rats, compound number 1.

The results suggest that the specific activity of the free thyroxine is at first higher in the thyroid than in the plasma and later becomes approximately equal in both. Thus, the free thyroxine in the thyroid gland would appear to be the precursor of that in the plasma. The passage of thyroxine from gland to plasma is facilitated by a concentration gradient of over 100:1.

It is concluded that thyroglobulin, after passing from the thyroid cell into the colloid, undergoes proteolysis with the release of its iodinated components. Of these, thyroxin (and possibly the unknown compound, number 1) is excreted into the plasma.

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MAINTENANCE OF PREGNANCY IN PYRIDOXINE-DEFICIENT RATS WHEN INJECTED WITH ESTRONE AND PROGESTERONE[1]

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PREVIOUS studies have shown that the addition of a pyridoxine antagonist (desoxypyridoxine) to a pyridoxine-deficient diet resulted in a high incidence of resorptions when normal adult rats were placed on such a diet only 10 to 20 days prior to breeding (Nelson and Evans, 1948, 1951). Supplementation with pyridoxine on the day of breeding counteracted the adverse effects of the antagonist. This was true even in pair-fed control animals for which the food intake averaged 56% of that consumed by control rats given the diet ad libitum (Nelson and Evans, 1951).

The early appearance of vaginal erythrocytes and the extreme rapidity of the resorptive process in these deficient rats together with the early loss of the estrous cycles in the unbred rats suggested the possibility of hormonal deficiencies in these animals. Lyons (1943) has shown that daily injections of 1 μg. estrone and 3–4 mg. progesterone will maintain pregnancy in hypophysectomized and oophorectomized rats. It was consequently thought desirable to test the efficacy of these hormones both singly and in combination in pyridoxine deficient rats.

EXPERIMENTAL METHODS

Stock rats of the Long-Evans strain were placed on the B_6-deficient diet containing the antagonist at 80–90 days of age. After 17 days of deficiency, the rats were bred with normal males and divided into equivalent groups as judged by body weight on the day of breeding. This experimental procedure has been shown previously to result in 90–100% resorptions of the deficient rats (Nelson and Evans, 1951). Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation had occurred. All rats were weighed at regular intervals. The progressive growth or resorp-

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tion of the uterine contents were checked frequently by gentle palpation. All rats were autopsied on the 16th-18th days of gestation[2]; implantation sites, fetuses, and ovaries were carefully examined. For some groups, implantation sites were measured (antero-posterior diameter) and the ovaries weighed and fixed for histological examination.

All injections were started 3–6 days after the day of breeding, i.e. the day of finding sperm in the vaginal smear. The hormones, estrone and/or progesterone, were injected subcutaneously in 0.2–0.3 cc. of sesame oil, and an equivalent amount of sesame oil was also given to one group. Since it was possible to breed only 50% or less of the deficient animals in any large group of 30 to 60 rats, four separate experiments were carried out. In each experiment one or two hormone-injected groups were studied together with an uninjected or sesame oil-injected group and usually a pyridoxine-supplemented group.

The B_6-deficient diet containing the antagonist was the same as that used previously (Nelson and Evans, 1948, 1951). It consisted of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco or Primex), 4% salts[3], and 0.5 mg.% desoxypyridoxine. Crystalline vitamins per kilogram of diet were: 300 μg. d-biotin, 5 mg. 2-methyl-1, 4-naphthoquinone, 5 mg. thiamine HCl, 5.5 pteroylglutamic acid, 10 mg. riboflavin, 10 mg. p-aminobenzoic acid, 20 mg. nicotinic acid, 50 mg. d-calcium pantothenate, 400 mg. inositol and 1.0 gm. choline chloride. 5 mg. pyridoxine HCl per kilogram was added to the diet for the control group. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units vitamin D, 6 mg. synthetic alpha-tocopherol, and 650 mg. corn oil (Mazola).

RESULTS

Table 1 summarizes the data from the four experiments. The deficient rats uninjected or injected with sesame oil showed 90% resorptions with the early appearance of erythrocytes in the vaginal smear (average day 10.8) and evidence of the extremely rapid resorptive process. Implantation sites were not observed in the majority of rats even though autopsies were performed on the 16th–18th days of gestation. The average number of implantation sites for these 26 rats was only 3.2 ± 0.9 per rat. These results may be compared with the pyridoxine-supplemented group, some of which were placed on the supplemented diet as late as 5 days after breeding. In this group 100% of the rats had living young at autopsy and erythrocytes appeared in the vaginal smear at the normal time (Day 12.6). The number of implantation sites and living young per rat were normal, i.e. 9.4 and 8.2, respectively. The gain in weight of 65 grams during the 17-day gestation period for the supplemented group is in marked contrast with the bare maintenance of weight for the deficient rats.

The injection of either estrone (1 μg. daily) or progesterone (4 mg. daily)

[2] It has been previously pointed out (Nelson and Evans, 1951) that the rapidity of the resorptive process in B_6-deficient rats is such that delaying the autopsy until the end of gestation resulted in a complete absence of implantation sites in many cases. It was thought that an earlier autopsy, i.e. 16th to 18th days, might yield more information on varying degrees of maintenance of the conceptus.

[3] Salts No. 4 of Hegsted *et al.* (1941).

TABLE 1. EFFECT OF ESTRONE AND PROGESTERONE ON REPRODUCTION OF PYRIDOXINE-DEFICIENT RATS

Exptl. group	Rats bred	Body wt. at mating	Wt. change during gestation	Vaginal RBC[1]	Implantations	Resorptions of implants	Litters of implants	Implantation sites	
								Av. No.	Normal No.[3]
	No.	gms.	gms.	day	%	%	%		
Pyridoxine-Deficient									
Uninjected	14	195	−3.0	10.9 ± 0.7[2]	86	92	8	2.8 ± 1.4	
Sesame Oil	15	197	+1.2	10.6 ± 0.6	93	93	7	3.5 ± 1.1	
1 μg. Estrone	10	194	+2.3	11.5 ± 0.2	80	87	13	6.5 ± 1.5	
4 mg. Prog.	10	182	−1.3	12.4 ± 0.3	100	90	10	7.4 ± 0.7	
1 μg. Estrone +4 mg. Prog.	9	194	+3.8	12.7 ± 0.5	100	11	89	8.1 ± 0.5	3.8 ± 0.9
2 μg. Estrone +4 mg. Prog.	10	195	+3.9	13.4 ± 0.7	90	78	22	8.0 ± 1.0	
3 μg. Estrone +4 mg. Prog.	10	212	+1.1	12.0 ± 0.8	90	56	44	8.8 ± 0.9	5.8 ± 1.9
6 μg. Estrone +4 mg. Prog.	10	212	+4.2	9.8 ± 0.4	100	90	10	9.0 ± 0.7	
Pyridoxine-Supplemented									
Uninjected	10	205	+64.5	12.6 ± 0.3	100	0	100	9.4 ± 0.2	8.2 ± 0.7

[1] This column shows the average time in days for the first appearance of erythrocytes in the vaginal smear.
[2] Standard deviation (error) of the mean.
[3] Values are given in the column only for groups in which at least 4 litters were obtained.

alone did not maintain pregnancy in the deficient rats. In the *estrone group*, the percentage of implantations was slightly less than normal and erythrocytes appeared slightly earlier than normal. Very small implantation sites (0.2 cm. average), composed principally of "metrial gland" remnants, were observed in the majority of the rats that implanted, thus increasing the number of implantation sites from that observed in the uninjected or sesame oil group to 6.5 per rat. A majority of the animals showed a cornified vaginal smear for 1–10 days after injections were started and half of the animals showed an estrous uterus at autopsy. In the *progesterone group* the percentage of implantations and the time of appearance of vaginal erythrocytes were in the normal range. Implantation sites were observed in all animals (7.4 per rat) and the size of the sites (0.4 to 1.4 cm.) was markedly increased in comparison with either the uninjected animals or the group injected with estrone.

The combined injection of 1 μg. estrone and 4 mg. progesterone was markedly beneficial. Pregnancy was maintained in 90% of the animals although there was no significant increase in body weight during gestation. The percentage of implantations and the time of erythrocyte appearance were normal as well as the number and size of the implantation sites. The number of living young per litter was, however, only about half the normal value, i.e., 3.8 vs. 8.2.

Pregnancy was maintained in only one-third of the deficient animals receiving a higher level, 2–3 μg., of estrone with 4 mg. progesterone and the number of living young per litter was not significantly increased. When the estrone level was increased to 6 μg. and given in combination with 4 mg. progesterone no maintenance of pregnancy was shown. The size of the implantation sites was greater and there was less failure of implantation than in rats injected with estrone alone. The exceedingly early appearance of vaginal erythrocytes before the 10th day in this group should be noted.

Ovarian weights for the deficient groups with or without hormone injections showed marked variability but no significant differences between groups. Histological studies of the ovaries from deficient rats in which resorption had occurred revealed degeneration of corpora lutea whereas these structures appeared essentially normal in deficient rats in which pregnancy had been maintained. This was regarded as evidence of leuteotrophic function of the placenta.

DISCUSSION

The data presented in this study demonstrate that pregnancy was maintained for at least 17 days[4] in 90% of the pyridoxine-deficient rats

[4] In a preliminary experiment, the injection of these hormones maintained pregnancy to term and normal parturition occurred.

by the daily injections of 1 μg. estrone and 4 mg. progesterone. Increasing the level of estrone (2–3 μg.) while maintaining the same level of progesterone was not as beneficial although pregnancy was maintained in 22–44% of the animals. A higher level of estrone (6 μg.) combined with 4 mg. progesterone was not beneficial nor was the injection of either hormone separately.

These results clearly indicate hormonal inadequacies of the pyridoxine deficient rat and demonstrate a relationship between the vitamin, pyridoxine, and the sex hormones, estrone and progesterone. This relationship could be concerned with (1) the pituitary production and secretion of gonadotropic hormones (FSH, ICSH, and lactogenic hormone) which control the ovary and cause secretion of estrone and progesterone; (2) the production and secretion of the ovarian hormones; (3) an increased requirement or a decrease in the sensitivity of the target organs, uterus and placenta, for the ovarian hormones; (4) the placental production of hormones; or (5) increased destruction or excretion of the pituitary, ovarian or placental hormones before utilization. Any combination of these factors could also be possible. In regard to the sensitivity of the target organs for the ovarian hormones the data presented show that the pyridoxine-deficient rat responded to the same levels of estrone and progesterone as the hypophysectomized-oophorectomized rat (Lyons, 1943). In addition, the majority of deficient rats injected with estrone alone responded with a cornified vaginal smear and some with an estrous uterus.

Under the experimental conditions, these B_6-deficient rats resemble the hypophysectomized-oophorectomized rat, i.e. pregnancy can be maintained by the combination of hormones but not by either hormone separately. The sex hormonal deficiencies of the B_6-deficient rat apparently are almost but not always as great as in the hypophysectomized-oophorectomized rat. This is indicated by the complete absence of implantation sites in hypophysectomized-oophorectomized rats either uninjected or injected with estrone in contrast to the occasional presence of implantation sites in the uninjected B_6-deficient rats and the presence of sites in the majority of B_6-deficient rats injected with estrone. An additional difference is the bare maintenance of weight in the B_6-deficient injected rats in which pregnancy is maintained in contrast to the gain in weight of the injected hypophysectomized-oophorectomized rats in which pregnancy is maintained (Lyons, 1943).

The individual variation in hormonal deficiency in this experimentally-timed B_6-deficiency is obviously great despite all efforts to standardize the animals. Approximately half of the rats lose their estrous cycles in the 3rd to 4th week of the deficiency and cannot be bred; these animals, therefore, are more deficient in hormones than the rats which have been bred during this period. A few animals have sufficient hormones for regular cycles as

judged by the vaginal smear technique but fail to breed due to lack of behavioristic estrus. The group of successfully bred rats have sufficient hormones to permit cycles, ovulation and implantation but not to permit maintenance of pregnancy except for approximately one rat in ten. This failure may be either the additional period of time on the deficient diet or the added demands of the conceptus. Mention has been made of the complete absence of implantation sites in the hypophysectomized-oophorectomized rat in contrast to the occurrence of implantation sites in approximately one-third of the deficient rats. These few rats with implantation sites presumably had slightly more circulating hormones than the rats in which no sites were observed. Likewise, 75% of the deficient rats injected with estrone alone must have had some progesterone present to maintain implantation sites in contrast to the remaining 25% of the group in which no sites were present.

Furthermore, in the bred rats injected with estrone and progesterone the products of conception were maintained to a varying degree. One rat in ten resorbed regardless of the presence of presumably adequate ovarian hormones. In the rats in which pregnancy was maintained the number and size of the implantation sites were normal but the number of living young per litter was less than half of the normal value. This failure may be explained on the basis of: (1) Excessive dosage or the wrong balance of estrin and progestin from three sources—injection, ovaries or placentae, (2) a deficiency of hormones other than those mentioned; or (3) an injurious effect of the vitamin deficiency on the placenta, fetus or some mechanism other than that of hormone production. In addition, the hormone injections did not maintain the normal gain in weight during gestation nor were any beneficial effects noted on the marked and specific acrodynia that develops rapidly in these deficient animals. Although the ovarian failure in these B_6-deficient rats has been corrected by administering the two female sex hormones, other symptoms of the deficiency have not been corrected.

Little can be said at present in regard to the specificity of this relationship of pyridoxine to the sex hormones during pregnancy. Injection of these two hormones, either separately or combined, have not maintained pregnancy in rats deficient in pantothenic acid or in pteroylglutamic acid[5]. Previous attempts to maintain pregnancy in vitamin E-deficient rats by these hormones but not in the combinations reported here have also failed, e.g. Ershoff (1943).

SUMMARY

Pregnancy was maintained to the 17th day of gestation in 90% of pyridoxine-deficient rats by injection of 1 μg. estrone plus 4 mg. progester-

[5] Nelson and Evans, unpublished experiments.

one daily but not by either hormone separately. In contrast, deficient rats uninjected or injected with sesame oil averaged only 10% maintenance of pregnancy under the same experimental conditions. Other combinations of estrone with progesterone were not as beneficial.

The results clearly indicate an inadequacy in the secretion of the ovarian hormones in pyridoxine deficiency. The B_6-deficient rat may be compared with the hypophysectomized-oophorectomized rat in which pregnancy is maintained by injection of the same hormones at the same levels.

Further work now in progress with pituitary, ovarian and placental hormones may throw more light on this vitamin-hormone interrelationship.

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PROGESTERONE OF THE BLOOD IN THE MENSTRUAL CYCLE OF THE MONKEY[1]

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ADVANCES in our knowledge of the physiology of estrous and menstrual cycles have been hampered by a lack of reliable, quantitative methods for the detection and assay of progesterone in small amounts of blood. Methods for the measurement of pregnandiol and other excretory metabolites of progesterone have been useful but are of limited quantitative value and are not suitable to all species of mammals (Pincus, 1948). The bio-assay technique recently devised by Hooker and Forbes (1947) seems to fulfill the requirements of a quantitative micro-method and at the same time is apparently specific for progesterone and thus obviating the presence of other steroid hormones that may be in the test material (Hooker and Forbes, 1949). Therefore, this procedure was used in the present investigation.

The purpose of this study was to determine the time of appearance and the amount of progesterone in the blood serum of normal adult monkeys (Macaca mulatta) during the menstrual cycle. In addition, observations were made on the effects of prolactin and chorionic gonadotropin (PU) on the functional life of the corpus luteum.

THE NORMAL MENSTRUAL CYCLE

Three mature monkeys that had just completed at least six normal menstrual cycles were used in the experiments. Blood samples were drawn from a superficial vein of the arm and the serum separated by centrifuging and stored in a deep freezing unit until used in the assay.

The assay technique employed closely followed that described by Hooker and Forbes (1947) with the exception that blood serum was used instead of an extract dissolved in sesame oil. In brief, the procedure consisted of the microinjection of .0006 ml. of a known dilution of serum into the lumen of an isolated segment of the uterus of a mouse that had been castrated at least sixteen days previously. Forty-eight hours later the animal was killed and the uterus removed for sectioning. A positive response

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being indicated by a characteristic reparative change in the atrophic nuclei of the stromal cells of the endometrium. The intra-uterine injections of serum diluted with normal saline were made until the minimal effective dose was obtained. The scale of dilutions employed was: undiluted serum, 1:3, 1:6, 1:18 and 1:30. It has been shown that the presence of 0.33 μg. of free progesterone per ml. of serum can be detected by this procedure.

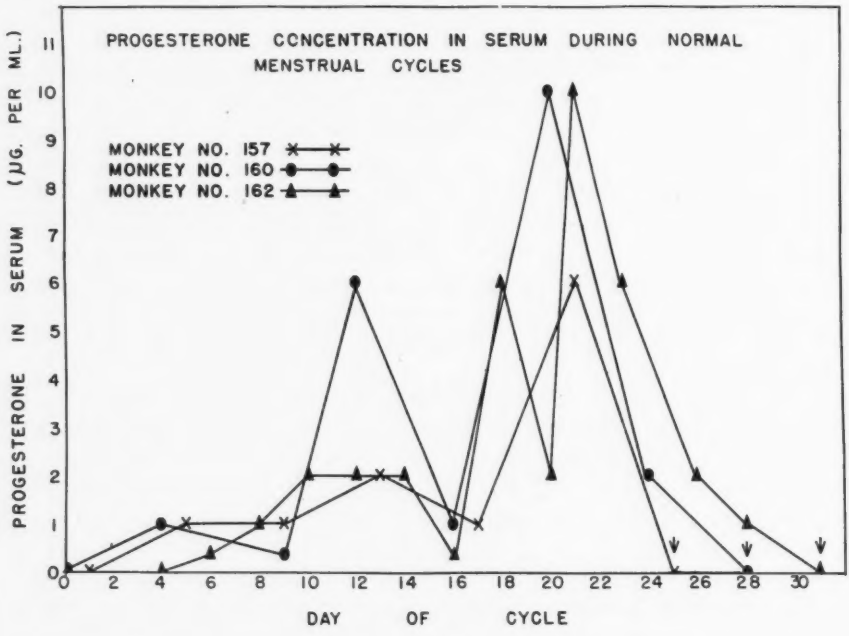

FIG. 1

The data obtained for the normal cycles of the three monkeys are in general agreement as to the time of appearance and disappearance of progesterone and its concentration in the blood at various times of the cycle (Fig. 1). The only exceptions to this are the relatively high concentration on the 12th day for monkey 160 and the relatively low concentration on the 20th day for monkey 162. In the case of 162, though the daily fluctuation of progesterone in the blood is not known, the wide difference between that of the 20th and 21st days suggests the possibility that the low reading on the 20th might have been due to a technical error. The variation on the 12th day for 160 is one of degree in that all three animals showed an increase in progesterone at this time of the cycle. The striking agreement between these results and those obtained by Forbes, *et al.* (1950) from a

similar study of the cycles of two monkeys indicates that probably these observations show at least the general character of the fluctuation of the concentration of progesterone in the blood during the menstrual cycle.

The presence of progesterone during the follicular half of the cycle is a point of considerable interest and especially its early appearance following menstruation. These data (Fig. 1) were supplemented by several assays for other animals and they indicate quite consistently that progesterone regularly attains a concentration of about 1 μg. per ml. of serum by the end of the first week of the cycle.

Another feature common to the three cycles is a decrease in progesterone on the 16th and 17th days. This apparently occurs shortly after ovulation. There seems no obvious explanation for this, though at this time the granulosa of the ruptured follicle must be undergoing transformation into luteal tissue and the associated rapid growth might interfere with secretion. This period of low concentration of progesterone is followed by an increase in each of the three animals until a maximum concentration is reached on the 20th or 21st day, after which there is apparently a more gradual decline, terminating in readings that show little or no progesterone in the blood at the beginning of menstruation.

PROLACTIN AND CHORIONIC GONADOTROPIN

The influence of these two luteotropic hormones on the functional life of the corpus luteum in monkeys has been reported (Hisaw, 1944) and advantage was taken of the present technique to follow the progesterone levels in the blood of animals given similar treatments. Four monkeys were used for this purpose, three of which were the same as those used in the studies on progesterone in the blood during the normal menstrual cycle. The presence of a functional corpus luteum in each animal was established by laparotomy at the beginning of the experiment between the 18th and 22nd day of the cycle. The hormones were dissolved in 0.9% saline no longer than 48 hours prior to injection. The dosage for the chorionic gonadotropin was 1000 I.U. given subcutaneously once daily while 400 I.U. of prolactin was injected subcutaneously in two divided doses morning and evening.

The menstrual cycles of the four animals given prolactin were not prolonged beyond their normal length, thus agreeing with the previous observations by Hisaw (1944). The progesterone of the blood rose from an initial low level at the beginning of the experiments to a maximal concentration corresponding approximately in time of occurrence to the peak levels seen in the three normal cycles though the readings were not quite as high (Fig. 2). The premenstrual fall seen in each case was also like that of the normal cycle though in one animal (164) bleeding occurred when the

blood progesterone level was 2 μg. per ml. It is worth mentioning that in this instance the menstrual blood loss was very slight, and was seen on only one day.

The injections of prolactin were started on the 18th and 19th day of the cycle which may account for the low levels of progesterone at the beginning of the experiments. These days correspond approximately with the time in

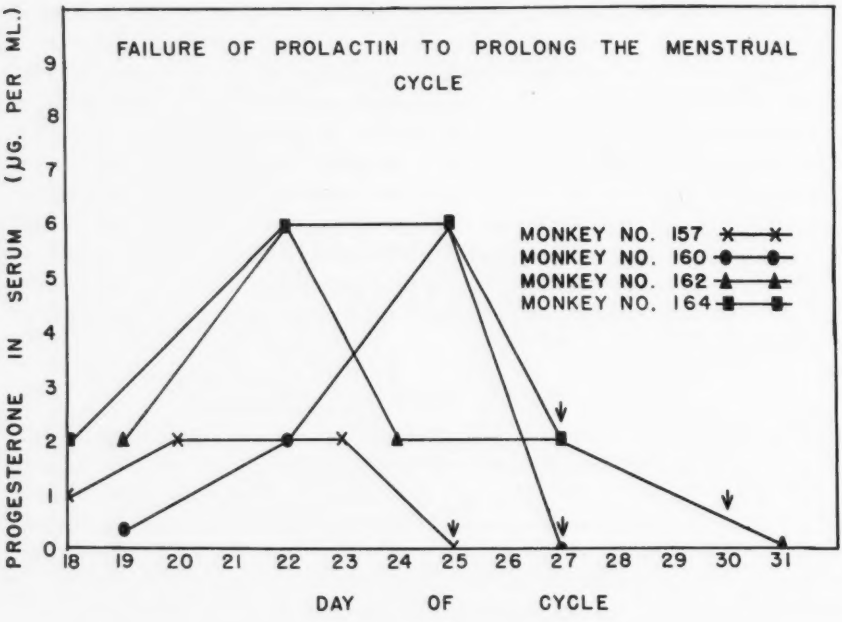

FIG. 2

the three normal cycles at which an apparent post-ovulatory decrease in progesterone takes place. It is of interest that all showed an increase of progesterone while receiving prolactin though for monkey 157 the two blood samples taken between laparotomy and bleeding indicate that it did not rise above 2 μg. per ml. The fact that corpora lutea were known to be present in these animals suggests a rather wide variability in luteal function. This monkey (157) also showed the least progesterone of the three animals used in the study of the normal cycle and less than monkey 160 when given chorionic gonadotropin.

In two animals, 157 and 162, taking of blood samples was continued following menstruation and cessation of injections of prolactin. In both instances, progesterone reappeared in the blood within five days after

bleeding. These observations are in agreement with those made in the studies of the normal cycles of untreated monkeys.

The menstrual cycles of the two animals given chorionic gonadotropin were definitely prolonged (Fig. 3). The experimental cycle of monkey 157 was 36 days and that of 160 was 32 days. The average length of the six previous cycles for these two animals was 26.2 days (157) and 25.6 days (160). The response of both animals was similar in that menstruation was

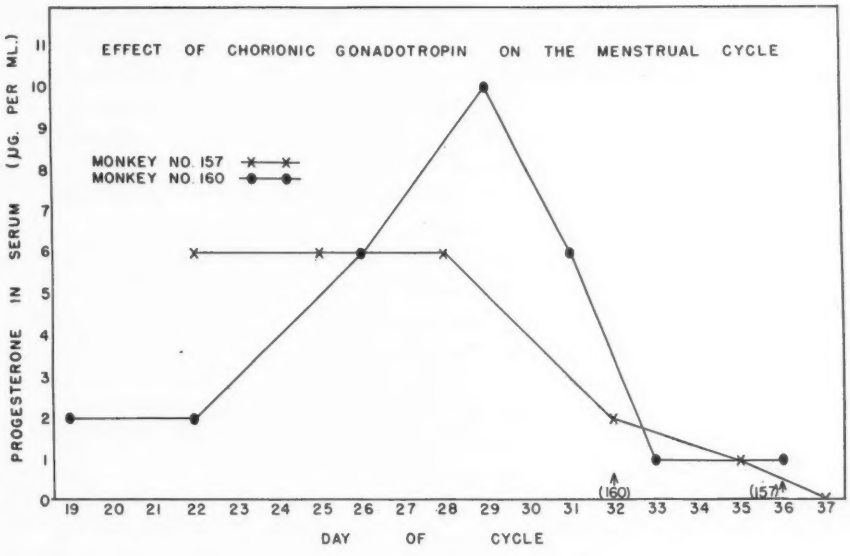

FIG. 3

postponed but they differed with regard to the amount of progesterone in the blood. Monkey 157 had 6 µg. of progesterone per ml. of serum when the injections of chorionic gonadotropin were started on the 22nd day of the cycle and this concentration was maintained until the 28th day. This animal also had the same amount of progesterone in the blood on the 21st day of the normal cycle (Fig. 1). Therefore, it seems probable that luteal function was at its maximum at the beginning of the experiment and continued at this level for about one week.

The reaction of monkey 160 is more difficult to explain. There were 2 µg. of progesterone per ml. of serum when the experiment was started on the 19th day while on the 20th day of the normal cycle (Fig. 1) there were 10 µg. per ml. The 19th day seems rather late for the post ovulatory decrease in progesterone as seen in the normal cycles yet it is possible that in this

instance ovulation might have been delayed two or three days. Some credence is given to this explanation by the fact that the corpus luteum evidently had not attained maximal capacity to secrete as shown by the subsequent increase of progesterone in the blood.

DISCUSSION

While it may be premature to contend that the data presented are quantitatively exact, it does seem reasonably certain that they do at least indicate the general fluctuation in concentration of progesterone in the blood during the normal menstrual cycle and under the experimental conditions investigated. This latter view is supported by the obvious fact that the maximal concentration of progesterone occurs in the blood during the luteal phase of the cycle, as should be expected, and is almost or entirely absent at the onset of menstruation. Also, as has been mentioned, there is agreement between these observations and those made independently by Forbes, Hooker, and Pfeiffer (1950).

The presence of progesterone during the follicular phase of the cycle probably should not be surprising as considerable evidence for its appearance previous to ovulation has been gathered for several species of mammals. Ring (1944) in mice, Boling and Blandau (1939) in rats and Dempsey *et al.* (1936) in guinea pigs, have shown that optimal sexual receptivity is conditioned by the synergistic action of progesterone and estrogen. In the rat, Astwood (1939), studying water content of the uterus, and Everett (1943), studying ovulation, have suggested release of progesterone during the period of pre-ovulatory follicular swelling. The cessation of uterine motility between the fifth and seventh hour after copulation in the rabbit can best be explained by the release of progesterone as was described by Reynolds and Friedman (1930).

In the human being Smith, Smith and Schiller (1942), studying urinary estrogen excretion, suggest that progesterone might be present as early as the 7th day of a 28 day cycle. Wilson *et al.* (1939) and, more recently, Davis and Fugo (1948) have reported the excretion of small but significant amounts of pregnandiol in women during the first part of the menstrual cycle. Hooker and Forbes (1947), using the bio-assay employed in this study, detected progesterone in the pre-ovulatory follicular fluid of the sow ovary.

Despite the rather impressive evidence that progesterone appears previous to ovulation, there is relatively little data on the anatomical basis of such secretion. However, the most probable source seems to be the ovary. The absence of progesterone in the blood of castrated animals, and normal animals during menstruation, as well as juvenile monkeys with a well developed sexual skin, speaks against an extra-ovarian source. On the other

hand, early luteal changes have been reported present in the walls of unruptured follicles in the late pre-ovulatory stage. Such observations have been made in the mouse (Allen, 1941), the rat (Boling *et al.* 1941), the cat (Dawson and Friedgood, 1940), the monkey (Corner, 1945), and in the mare (Seaborn, 1925). Therefore, it seems most likely that the presence of progesterone in the blood as early as the first week of the menstrual cycle can best be explained by the assumption that the follicle attains a degree of competence to secrete progesterone previous to ovulation and definite luteinization.

The significance of the decrease in progesterone on the sixteenth and seventeenth days is difficult to assess. This drop apparently coincides with the early post-ovulatory period. A possible explanation is that at this time the granulosa of the ruptured follicle must be undergoing transformation into luteal tissue and the associated rapid growth might interfere with secretion.

That the secretory life of the corpus luteum is prolonged under the influence of injections of chorionic gonadotropin is demonstrated by the maintained levels of progesterone beyond the normal limit of the cycle. This corroborates earlier observations (Hisaw, 1944) on monkeys similarly treated in which a lengthening of the cycle was associated with progestational endometria and other luteal effects.

The amounts of progesterone indicated as being present at different times of the menstrual cycle probably should not be considered as quantitatively exact until more is known regarding the accuracy of the assay method as used in these experiments. However, there seems less reason to doubt the qualitative significance of the data and if this technique proves as specific for progesterone as it now seems it will be a valuable aid in studies of ovarian physiology and related problems.

SUMMARY

The concentration of progesterone in the blood during normal and experimental menstrual cycles in monkeys was determined by the bio-assay method devised by Hooker and Forbes. There was general agreement between results obtained from studies of normal cycles of three animals. Progesterone disappeared from the blood either immediately before or during menstruation and reappeared in detectable amounts within the first week of the cycle. This was followed by an increase in concentration toward the end of the follicular phase at a point corresponding approximately with the time of expected ovulation. This rise was followed by a decline until about the 16th or 17th day which was succeeded by a rapid increase until the maximal concentration of progesterone was attained near the middle of the luteal phase. During the last half of the luteal phase

the amount of progesterone in the blood decreased and was usually absent at the beginning of menstruation.

The secretory life of the corpus luteum was not prolonged in four monkeys that were given prolactin during the luteal phase of the cycle. Menstruation occurred at the normal time and was preceded by the usual decrease of progesterone in the blood.

The menstrual cycles of two monkeys given chorionic gonadotropin were prolonged seven and ten days beyond the expected normal duration. That this was due to a prolongation of luteal function was indicated by the continued presence of progesterone in the blood.

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THE INFLUENCE OF THE ADRENAL CORTEX ON ANTIBODY PRODUCTION *IN VITRO*[1]

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INVESTIGATIONS of the possible influence of the adrenal cortical secretions on the production and release of antibodies *in vivo* have produced conflicting results. It has been reported that adrenalectomy prior to immunization reduces (Dougherty, Chase, and White, 1945), increases (Murphy and Sturm, 1947), or fails to alter (Eisen *et al.*, 1947; Thatcher, Houghton, and Ziegler, 1948; Stoerk and Solotorovsky, 1950) the subsequent course of the immune response. Similar lack of agreement exists among different investigators with respect to the influence of adrenal cortical hormones or of adrenocorticotrophic hormone (ACTH) on antibody production. An increase in circulating antibody after administration of these hormones has been observed by Fox and Whitehead, 1936; Dougherty, White, and Chase, 1944; Dougherty *et al.*, 1945; Chase, White, and Dougherty, 1946; Mondolfo and Hounie, 1947; Bisset, 1949; Bussard *et al.*, 1950; Clark and Jacobs, 1950; and Hammond and Novak, 1950. On the other hand, a number of workers have been unable to confirm these results (Eisen *et al.*, 1947; Thatcher *et al.*, 1948; Kelley and Adams, 1948; Sayers *et al.*, 1949; Fischel, Le May, and Kabat, 1949; Herbert and de Vries, 1949; de Vries, 1950; Mirick, 1950). One group of investigators has reported a decrease in circulating antibody after ACTH or cortisone injection (Stoerk, 1950; Stoerk and Solotorovsky, 1950; Bjørneboe *et al.*, 1951).

The results of the studies reported below suggest that blood titers of antibody may not reflect accurately the level of antibody response in animals exhibiting different levels of adrenal cortical activity. Thus, it can be demonstrated that splenic production of antibody *in vitro* may be significantly affected by adrenal cortical secretions without necessarily influencing appreciably the level of circulating antibody. This may be due, at least in part, to a coincident change in the rate of antibody removal from the blood by the tissues as a result of changes in the circulating level of adrenal cortical hormone.

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METHODS

The procedures employed in this investigation have been described in considerable detail elsewhere (Roberts, Adams, and White, 1949) and will be recorded here only briefly.

The animals used were adult male Sprague-Dawley rats, weighing between 250 to 300 gm., and maintained *ad libitum* on Purina laboratory chow. Bilateral adrenalectomy was performed by the dorsal approach 2 to 4 days before antigen injection. Adrenalectomized rats were given 1 per cent NaCl to drink instead of the tap water allowed the other animals. Immunization, in all instances, was accomplished by a single intravenous injection of 0.5 ml. of a 2 per cent suspension of sheep erythrocytes, delivered into the saphenous vein under light ether anesthesia.

Serum and tissue antibody were estimated by hemolysin titrations employing the doubling-dilution technique. Tissue antibody was determined on saline extracts by grinding 200 mg. aliquots of tissue with sand and 2 ml. of physiological saline, followed by centrifugation in the cold to remove the solid material. All titers reported in this presentation are expressed as the reciprocal of the hemolysin titer per ml. of serum or per gm. of tissue extracted or incubated. Expressed in this manner, the values are presumed to be directly proportional to the antibody content of the material being assayed. Differences equal to or greater than 2 tubes in the doubling-dilution technique (i.e., differences of 200 per cent or greater) may be considered significant, if obtained consistently.

All *in vitro* studies were conducted in Warburg flasks, containing 200 mg. of tissue mince (consisting mainly of intact cells) and 2 ml. of fresh rat serum. Incubation was carried out for 3 hours at 38° C., with a constant stream of 95 per cent oxygen, 5 per cent carbon dioxide passing through the flasks.

RESULTS

1. *Influence of the adrenal cortex on the development of serum and tissue hemolysin titers*

Preliminary to the *in vitro* studies on the release and uptake of antibody, experiments were performed to establish, after antigen injection, the pattern of antibody development with time in the serum and lymphoid structures of control rats, rats which had been bilaterally adrenalectomized 2 to 4 days prior to antigen injection, and rats which had been treated with Wilson's aqueous adrenal cortical extract (A.C.E.) during the period of antibody development. Beginning on the day of immunization, the animals in the last group were given single subcutaneous injections daily for 3 days of 2 ml. of A.C.E.

The results obtained are shown in Figure 1. The time interval chosen for antibody determinations was the early period of 3 to 7 days after antigen administration, during which, in control animals, the serum hemolysin titer reaches a maximum and then declines gradually (cf. Roberts, Adams, and White, 1949). The maximum titer was observed in most tissues on the fifth day after immunization. This has been shown previously for a wide range of tissues, as well as serum, and was found to be the case in the

present study for serum, spleen, mesenteric lymph nodes, and thymus in control, adrenalectomized, and A.C.E.-injected rats.

Some differences in the rate of antibody development appeared to have occurred, particularly between adrenalectomized and control animals. The rate of development of antibody titers in serum and lymphoid tissues may have been depressed by previous adrenalectomy. The differences ob-

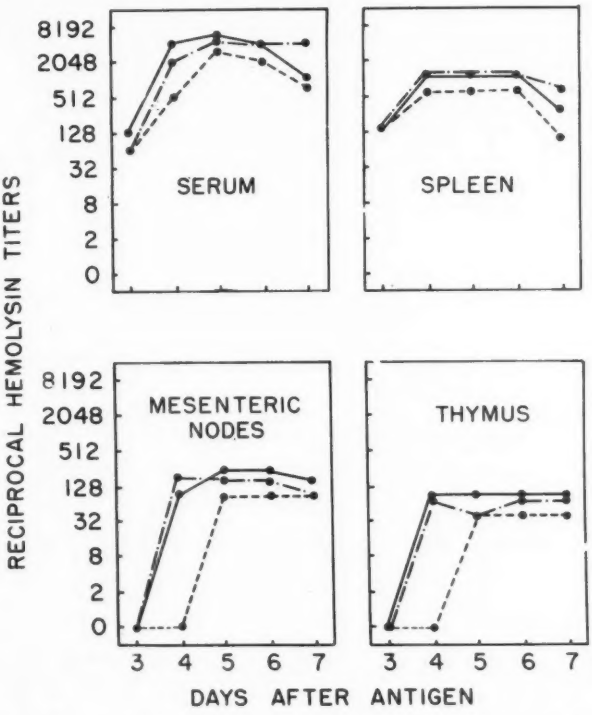

FIG. 1. The influence of the adrenal cortex on the development of hemolysin titers in serum and tissues of rats given a single intravenous injection of sheep erythrocytes. The solid lines represent control animals; dotted lines, adrenalectomized; dashed lines, A.C.E.-injected. Each point is the average of 3 to 5 determinations.

served, however, among the 3 groups could not be considered significant, with two possible exceptions. First, it may be seen in Figure 1, that adrenalectomy prevented the rapid rise in antibody in mesenteric lymph nodes and thymus tissue, which occurred in non-operated animals on the fourth day after antigen injection. This difference was noted consistently and may have been due to a reduction in the rate of uptake of antibody from the circulation in the adrenalectomized animal (see section 3, Results). The

TABLE 1. THE INFLUENCE OF ADRENAL CORTICAL ACTIVITY ON THE HEMOLYSIN TITERS OF SERUM AND SPLENIC AND LYMPHOID TISSUE EXTRACTS OF IMMUNIZED RATS*

Group	Serum	Spleen	Mesenteric lymph nodes
Control	2,048	640	320
	2,048	640	320
	8,192	640	320
	8,192	1,280	320
	8,192	1,280	320
	8,192	1,280	320
	16,384	2,560	640
	16,384	2,560	320
	4,096	1,280	—
	4,096	640	—
	4,096	1,280	40
	4,096	1,280	40
Average	6,144	1,280	240
Adrenalectomized†	2,048	640	80
	2,048	320	80
	4,096	640	80
	4,096	640	80
	4,096	640	80
	2,048	640	160
	4,096	640	160
	4,096	1,280	160
	4,096	—	—
	4,096	—	—
Average	4,096	640	120
Hormone-treated‡	8,192	1,280	160
	8,192	1,280	80
	8,192	640	160
	8,192	1,280	160
	8,192	1,280	320
	8,192	1,280	160
	4,096	1,280	160
	4,096	1,280	160
Average	8,192	1,280	160

* Hemolysin titers are expressed as reciprocals of the actual titer per ml. of serum or per gm. of tissue extracted, obtained 5 days after a single intravenous injection of sheep erythrocytes.

† Bilaterally adrenalectomized 7 to 9 days earlier.

‡ Intraperitoneal injection 2 hours earlier of either 2 ml Wilson's aqueous adrenal cortical extract or adrenocorticotropic hormone (Armour) equivalent to 4 mg. (Armour standard) LA-1-A per 100 gm. body weight.

second significant difference was the prolongation of maximum serum hemolysin levels beyond the fifth day in rats injected with A.C.E.

In the normal animal, the peak of antibody production by splenic tissue *in vitro* occurs on the fifth day after intravenous administration of antigen (Roberts, Adams, and White, 1949). It was, therefore, decided to investigate in greater detail the hemolysin titers present at this time in the serum and in extracts of spleen and mesenteric lymph nodes obtained from animals treated in a manner similar to that described above. Thymus tissue extracts were not followed in subsequent experiments since the titers ob-

served in thymus were entirely similar to those present in the lymph nodes (cf., Figure 1). The results are shown in Table 1. No significant differences were noted among the three groups, although the serum and tissue titers in the adrenalectomized animals tended to be consistently lower than similar values in the other animals. Incidentally, the hormone-treated group, in this case, was injected intraperitoneally 2 hours before tissue and serum sampling either with 2 ml. of A.C.E. or with a dose of ACTH (Armour) equivalent to 4 mg. of the Armour standard, LA-1-A per 100 gm. body weight.

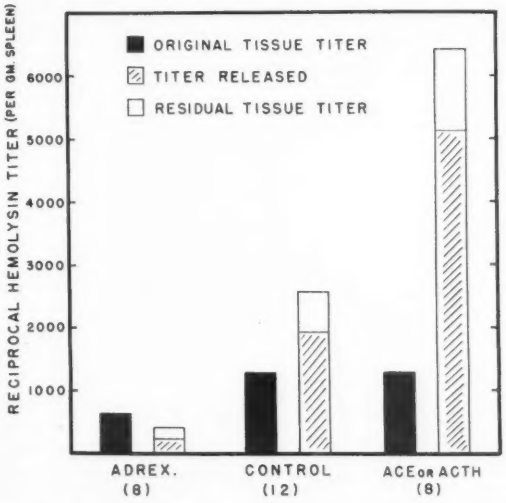

FIG. 2. The influence of the adrenal cortex on the release of hemolysins in vitro by splenic mince obtained from rats given a single intravenous injection of sheep erythrocytes 5 days earlier. The figures in parentheses show the number of experiments in each group contributing to the average.

2. *Influence of the adrenal cortex on splenic release of hemolysins in vitro*

In contrast to the results obtained on the effect of the adrenal cortex on the level of circulating and tissue antibody *in vivo*, a striking influence of the adrenal cortex was observed when studies were made of the capacity of surviving splenic tissue to produce antibody *in vitro*. Figure 2 depicts the data graphically. The spleen was chosen since it has been found to be the only tissue capable of releasing large amounts of antibody *in vitro* when taken from animals immunized by a single intravenous injection of antigen (Fagraeus, 1948; Roberts, Adams, and White, 1949).

Apparently, the spleen of the adrenalectomized rat was totally incapable of new formation of antibody under these conditions, whereas that of the

TABLE 2. THE INFLUENCE OF ADRENAL CORTICAL ACTIVITY OF THE RELEASE OF HEMOLYSINS *in vitro* BY SPLENIC TISSUE OF IMMUNIZED RATS*

Group	Incubation release† (1)	Post-incubation extraction (2)	Incubation + post-incubation (3)	Pre-incubation extraction (4)	Ratio $\frac{(3)}{(4)}$‡
Control	640	320	960	640	1.5
	640	320	960	640	1.5
	2,560	320	2,880	640	4.5
	2,560	1,280	3,840	1,280	3.0
	2,560	1,280	3,840	1,280	3.0
	2,560	1,280	3,840	1,280	3.0
	2,560	1,280	3,840	2,560	1.5
	2,560	1,280	3,840	2,560	1.5
	2,560	640	3,200	1,280	2.5
	1,280	640	1,920	640	3.0
	2,560	640	3,200	1,280	2.5
	2,560	640	3,200	1,280	2.5
Average	1,920	640	2,560	1,280	2.5
Adrenalectomized§	160	40	200	640	0.3
	80	40	120	320	0.4
	320	320	640	640	1.0
	320	320	640	640	1.0
	320	320	640	640	1.0
	320	320	640	640	1.0
	320	160	480	640	0.8
	320	160	480	1,280	0.4
Average	320	160	480	640	0.7
Hormone-treated‖	10,240	1,280	13,520	1,280	10.5
	2,560	1,280	3,840	640	6.0
	5,120	1,280	6,400	1,280	5.0
	5,120	640	5,760	1,280	4.5
	5,120	1,280	6,400	1,280	5.0
	5,120	1,280	6,400	1,280	5.0
	10,240	1,280	13,520	1,280	10.5
	5,120	640	5,760	1,280	4.5
Average	5,120	1,280	6,400	1,280	6.4

* Hemolysin titers are expressed as reciprocals of the actual titer per ml. of serum or per gm. of tissue extracted or incubated; samples were obtained 5 days after a single injection of sheep erythrocytes.

† Incubation was carried out for 3 hours in a rat serum medium at 38° C., and under a constantly renewed atmosphere of 95% oxygen, 5% carbon dioxide.

‡ The average of these ratios in each group was calculated from the individual values.

§ Bilaterally adrenalectomized 7 to 9 days earlier.

‖ Intraperitoneal injection 2 hours before autopsy of either 2 ml Wilson's aqueous adrenal cortical extract or adrenocorticotropic hormone (Armour) equivalent to 4 mg. (Armour standard) LA-1-A per 100 gm. body weight.

hormone-treated rat was very active in this regard. Control spleen fell somewhere between these two groups in its capacity to produce antibodies *in vitro*. A résumé of the *in vitro* results obtained is given in Table 2. The last column in this table is a measure of the production of antibody, since it relates the amount of antibody present after incubation to that present before (i.e., the ratio of the sum of the reciprocal hemolysin titer released to the medium plus that remaining in the tissue after incubation, to that

which can be extracted before incubation from the aliquot of the same tissue). This ratio was approximately 1 in the adrenalectomized group, indicating that no new formation of antibody occurred during the three-hour incubation period. Two-and-one-half times as much antibody was present after incubation as before incubation in the control group, and almost six-and-one-half times the initial amount was present in incubates of spleen obtained from A.C.E. or ACTH-treated rats.

3. *Influence of the adrenal cortex on antibody uptake in vitro.*

In an effort to elucidate the discrepancy between the *in vivo* and *in vitro* results with respect to the influence of the adrenal cortex on antibody production, two possibilities were considered. First, it seemed possible that differences in the rate of antibody production by the tissues might be masked in the circulation, particularly at times when the circulating titer is high. A second possible explanation seemed to be amenable to experimental study; namely, that the rate of removal of antibody from the blood

TABLE 3. THE INFLUENCE OF ADRENAL CORTICAL ACTIVITY ON THE UPTAKE OF ANTIBODY FROM IMMUNE SERUM BY SURVIVING TISSUES OF NON-IMMUNIZED RATS*

Tissue	Group	Serum medium		Tissue extracts after incubation†
		Before incubation	After incubation	
Thymus	Control	4,096	2,048	160
	Adrenalectomized‡	4,096	4,096	40
	Hormone-treated§	4,096	2,048	320
Mesenteric lymph nodes	Control	8,192	4,096	320
	Adrenalectomized	8,192	8,192	80
	Hormone-treated	8,192	2,048	640
Liver	Control	4,096	2,048	1,280
	Adrenalectomized	4,096	4,096	320
	Hormone-treated	4,096	1,024	2,560
Upper jejunum	Control	4,096	2,048	320
	Adrenalectomized	4,096	4,096	160
	Hormone-treated	4,096	2,048	640
Kidney	Control	4,096	4,096	1,280
	Adrenalectomized	4,096	4,096	640
	Hormone-treated	4,096	2,048	2,560
Spleen	Control	8,192	4,096	640
	Adrenalectomized	8,192	8,192	320
	Hormone-treated	8,192	4,096	640

* All results are expressed as reciprocal hemolysin titers per ml. of serum or per gm. of tissue. The averages of three experiments are shown in each case.

† After incubation, the tissue minces were washed with physiological saline prior to extraction of antibody; no titer was present in any of the tissues before incubation.

‡ Bilaterally adrenalectomized 7 to 9 days earlier.

§ Intraperitoneal injection 2 hours earlier of either 2 ml. Wilson's aqueous adrenal cortical extract or adrenocorticotropic hormone (Armour) equivalent to 4 mg. (Armour standard) LA-1-A per 100 gm. body weight.

as well as the rate of production, might be affected by the state of adrenal cortical activity.

It had been observed previously that tissues from non-immunized rats are capable of removing antibody from an incubation medium consisting of serum obtained from immunized animals (Roberts, Adams, and White, 1949). The generalized distribution of antibody in most of the tissues of the immunized rat, and its more rapid rate of accumulation in certain tissues when the antibody level is falling after the peak circulating level has been reached, had suggested that this might be the case. Accordingly, the uptake of antibody by tissues from control, adrenalectomized, and A.C.E. or ACTH-injected, *non-immunized* rats was followed. These tissues were incubated as described earlier, with the exception that the incubation medium was antibody-containing serum obtained from similar control animals which had been given a single intravenous injection of sheep erythrocytes 5 days earlier.

It may be seen from Table 3 that all of the tissues studied were capable of removing antibody from the serum medium; incubation of serum alone had no significant effect on its titer. The antibody taken up by the tissues was rather firmly fixed since it could not be removed by washing with saline or serum, but only after grinding the tissue with sand and saline. Adrenalectomy depressed the uptake of antibody by the tissues studied, particularly by thymus, mesenteric lymph nodes, and liver. A.C.E. or ACTH appeared to have the opposite effect, although this was not as clear-cut. The possible significance of these observations, with particular reference to the role of liver and lymphoid tissue in removing circulating antibody, will be discussed below.

DISCUSSION

The results reported indicate that the rate of antibody production *in vitro* by surviving splenic tissue from rats injected with a single intravenous dose of sheep erythrocytes may be depressed by previous adrenalectomy and enhanced by injection of pituitary adrenocorticotrophic or adrenal cortical hormones.

In spite of these differences in the *in vitro* behavior of the spleen (which is apparently the only tissue producing large amounts of antibody under these conditions), there appeared to be no significant differences in the level of *circulating* antibody which could be related to changes in the level of adrenal cortical hormones in the body.

Two probable explanations for this discrepancy may be suggested. First, it seems likely that differences in the rate of tissue production and release of antibody may be masked by the relatively enormous quantities of antibody present in the circulation of the immunized animal at or near the

peak of antibody response. A second explanation is based on the observation that the removal of antibody from the circulation may also be affected by the level of adrenal cortical secretion, being depressed in adrenalectomized rats and possibly enhanced in rats treated with A.C.E. or ACTH (cf., also, Stoerk, 1950). In other words, release and removal of antibody may both be occurring at a reduced rate in the adrenalectomized animals and at an accelerated rate in the hormone-treated animal. The balance achieved with respect to the level of circulating and tissue antibody may, therefore, be similar in the three groups of animals. It seems likely that one organ which may be responsible for removing large amounts of antibody from the circulation, and which may be particularly responsive to cortical hormone, is the liver. The rate of antibody uptake by this organ *in vitro* was significantly influenced by previous adrenalectomy. The uptake of antibody *in vitro* from immune serum by mesenteric lymph nodes and thymus tissue was also significantly altered by this procedure. The particular responsiveness of protein metabolism in lymphoid tissue to adrenal cortical hormones has been demonstrated earlier (cf., White and Dougherty, 1946; White and Roberts, 1950). It has been suggested that lymphoid tissue may function specifically as a carrier of antibody (Dougherty, Chase, and White, 1945). From the present studies, it would appear that its efficiency as such a transport mechanism may be under the control of the adrenal cortex, which may influence not only the rate of release of antibody from lymphocytes (Dougherty, Chase, and White, 1945), but also the amount of antibody which can be taken up by lymphocytes when other reticulo-endothelial elements are the major site of antibody formation.

The influence of the adrenal cortex on the metabolism of antibody *in vitro* may represent but one instance of a more general phenomena. Recent studies have revealed that several tissues, when incubated in a homologous serum medium, are capable of adding and removing specific proteins during the course of incubation (Roberts and White, 1949; White and Roberts, 1950). These processes have been found to be under the influence of the adrenal cortex (White and Roberts, 1950). Such changes in the *in vitro* metabolism of protein by specific tissues may or may not be reflected in variations in the level of circulating plasma proteins in the intact animal (cf., White and Dougherty, 1946; Gutman, 1948). The *in vivo* experiments of Whipple and his co-workers (cf., Madden and Whipple, 1940), suggesting the existence of a dynamic interchange of protein between tissues and plasma, find their *in vitro* counterpart in the studies mentioned above. In addition, the latter investigations indicate that this dynamic interchange may be influenced in rate by pituitary-adrenal cortical secretion.

SUMMARY

Investigations are reported of the influence of adrenalectomy and of injection of adrenocorticotrophic hormone and adrenal cortical extract on antibody development in tissues and serum *in vivo*, antibody release by splenic tissue *in vitro*, and the uptake of antibody by tissues *in vitro*.

In general, the rate of development of hemolysin titers in the tissues and serum of rats injected with a single intravenous dose of sheep erythrocytes could not be altered significantly by changes in the level of circulating adrenal cortical hormones. A possible exception to this may be seen in the delay in the development of high antibody titers in lymphoid tissue (mesenteric lymph nodes and thymus) in the adrenalectomized animal on the fourth day after antigen administration. This difference, however, appears to have disappeared by the fifth day, when most tissues and serum exhibit the peak of antibody response.

In contrast, the rate of release of antibody to a serum medium *in vitro* by splenic tissue was significantly affected by the degree of adrenal cortical activity. No new formation of antibody occurred in incubates of splenic tissue obtained from adrenalectomized animals. The production of antibody by splenic tissue *in vitro* was enhanced over the control value in animals injected 2 hours earlier with potent adrenal cortical extract or pituitary adrenocorticotrophic hormone.

The discrepancy between the *in vivo* and *in vitro* results may be explained by the observation that antibody uptake by various tissues *in vitro*, particularly by liver and mesenteric lymph nodes, may be depressed by previous adrenalectomy and, possibly, enhanced by injection of hormone. This would obviously tend to make the level of circulating antibody a false index of the immune response. It may also be suggested that differences in antibody production, particularly at the peak of antibody response, would be masked by the high levels of antibody already present in the circulation.

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ANDROGEN INFLUENCE ON PITUITARY-GONAD INTERRELATIONSHIP

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THE complex problem of pituitary-gonad interrelationship has attracted renewed attention because of the interest in the action of target-organs as modifying factors in this interrelationship. The complexity of the problem and the fact that adequate information for comparisons is not available has been reviewed in an excellent fashion by Greep and Jones (1950). Our own interest in the problem was stimulated by observations which we made on chicks and baby capons (Breneman 1950, 1951a, 1951b); and by some unpublished observations which in part suggested that the gonads as target-organs were important in the interrelationship. Some of the data on the other hand, could be best interpreted by accepting the traditional viewpoint that the androgens directly inhibit the pituitary.

We began the experiments reported here before the publication of the work of Greep and Jones. It was very gratifying, however, to note many important similarities in procedures which make comparisons possible. Although we used chicks whereas Greep and Jones used rats, the injection methods were comparable. Testosterone-propionate was the hormone used in both studies and was injected during the period in which the experimental animals were undergoing their most rapid growth and maturation. The hormone was injected subcutaneously in 0.10 cc. of sesame oil daily (including Sundays) into animals of uniform age in both experiments. We have found that chicks and baby capons are especially sensitive to testosterone-propionate and although some of our dosages of hormone were lower than those of Greep and Jones, the dosage ranges were similar and overlapped. They used a daily dosage in male rats of: none, 10 gamma, 20 gamma, 50 gamma, and 100 gamma whereas we used daily dosages in capons of: none, 0.5 gamma, 1.0 gamma, 5.0 gamma, 10 gamma, and 50 gamma.

The comb-growth of the chick measured either daily or at intervals of approximately five days is an excellent indicator of the effect of both

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exogenous and endogenous androgen in the chick. In this respect the chick or capon is more informative than the male mammal in which only the weight of the prostate or seminal vesicles taken at the termination of an injection period can serve to estimate androgen action. Comb weights which were taken at autopsy, however, gave us information comparable to that furnished by prostate weight. We have also been able to secure information on the weight and gonadotropic potency of the pituitaries, and pituitary glands were also fixed for cytological study.[3]

MATERIAL AND METHODS

Single-comb White Leghorn chicks were used in our experiments. These animals were one-day old when received and were kept in battery brooders throughout the period of the experiments. A standard starting-mash was fed and the number of birds in each cage was kept approximately equal. The position of the birds in the batteries was also rotated to eliminate possible effects due to differences in lighting or in temperature between top and bottom cages. The chicks which were caponized were operated when five days old. Injections were begun on the 10th day and continued through the 39th and all animals were killed on the 40th day. Comb measurements were taken on the 10th, 16th, 21st, 26th, 31st, 35th, and 40th days and a *comb-factor* represented by $\frac{\text{Height} \times \text{Length mm.}}{2}$ was determined. At autopsy the combs were removed by cutting parallel to the head with fine scissors. Combs under one gram were weighed on a torsion balance and those over one gram were weighed on a beam balance.

The anterior lobe of the pituitary gland was removed immediately and the glands from each series were placed in a moist chamber and weighed as a group. The pituitaries were then stored in acetone and at the time of assay were dried, ground fine in a mortar, taken up in distilled water, and injected on the basis of 4 mg. equivalent of fresh gland in one cc. of water. A total of five injections were given spaced at 12 hour intervals beginning at 18 hours post-hatching. The chicks were kept in shipping boxes without food or water during the injection period and were autopsied 12 hours after the final injection. This method of assay which utilizes the testis weight of the chick to estimate the amount of gonadotropins was first suggested by Byerly and Burrows (1938) and has proven to be very successful in our experience with pituitary assay (Breneman, 1945).

As was previously stated testosterone-propionate (T.P.)[4] was the androgen employed in this study. Injections were made subcutaneously into capons with each animal receiving 0.1 cc. of sesame oil daily as the carrier for the hormone. The males and control capons received the same amount of sesame oil without the hormone. The pullets were not injected.

RESULTS AND DISCUSSION

Comb-growth: The chick comb is an excellent indicator of androgen action and since measurements of the combs were taken at both the start

[3] The authors wish to express their sincere thanks to Dr. Fernandus Payne for his careful study of the cytology of the pituitary glands.

[4] The testosterone-propionate used in this investigation was furnished through the kindness of Dr. Edward Henderson, Director of the Schering Corporation, Bloomfield, New Jersey.

and termination of the experiments and at intervals between it is possible to gain a surprising amount of information concerning androgen action. Especially noteworthy are the data which supply clues as to the amount of androgen secreted by the male. Various methods of treating the data statistically were employed and the most informative was the relationship between the logarithm of comb-factor (and weight) against the logarithm of dose. This resulted in a straight-line relationship which was remarkably uniform as subsequent reference to Graph 1 will illustrate. This treatment also has been discussed briefly by Walters (1950). The mean comb-factors for the various days and dosages are summarized in Table 1 and a statement should be made relative to the method of calculating these means.

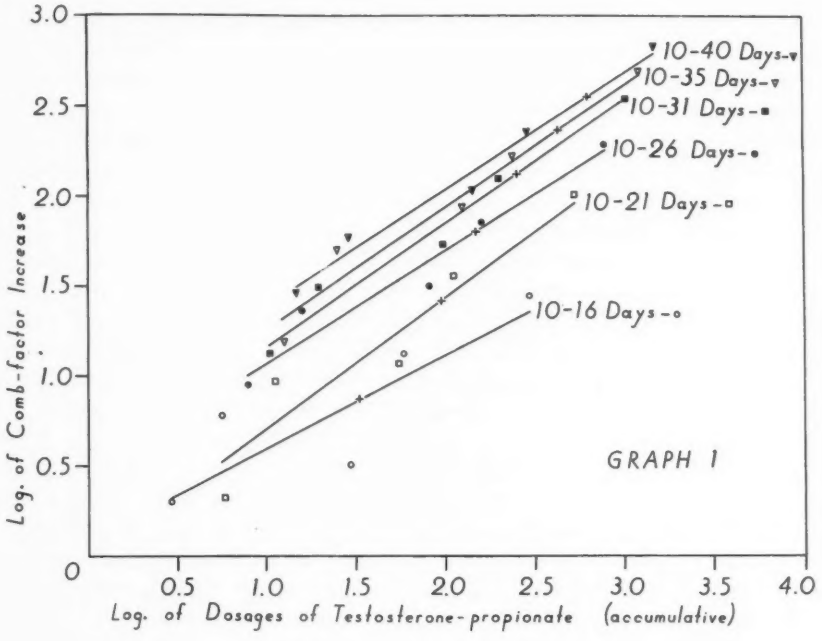
TABLE 1. ANALYSIS OF COMB-FACTOR (H×L mm./2) INCREASES* FROM 10TH TO 39TH DAY

Day	♀	♀ +0.5γ	♀ +1.0γ	♀ +5.0γ	♀ +10.0γ	♂ +50.0γ	♂	Total† androgen secretion by ♂	Cumulative daily average	Interval daily average
10										
Initial size	9.64	10.18	10.45	9.45	9.17	10.73	10.78			
16										
Period size	17.54	19.86	24.23	20.54	30.29	46.41	26.12			
Increase*	7.90	1.78	5.88	3.19	13.22	27.78	7.44	31.74γ	5.29γ	5.29γ
21										
Period size	33.77	36.41	43.95	45.23	69.71	135.18	60.62			
Increase	24.13	2.10	9.37	11.65	36.41	100.32	25.71	94.56γ	8.60γ	12.56γ
26										
Period size	56.77	66.36	80.27	87.73	125.50	251.17	122.87			
Increase	47.13	9.05	22.69	31.15	68.90	193.28	64.96	155.60γ	9.72γ	12.21γ
31										
Period size	80.00	94.00	113.27	135.86	207.50	425.59	212.78			
Increase	70.36	13.46	32.46	56.05	127.97	344.50	131.64	250.80γ	11.94γ	19.04γ
35										
Period size	91.63	107.86	142.77	176.90	256.12	577.95	328.81			
Increase	81.99	15.69	50.33	85.46	164.96	485.23	236.04	437.20γ	17.49γ	46.60γ
40										
Period size	125.59	155.81	185.59	235.36	355.54	801.95	479.62			
Increase	115.95	29.68	59.19	109.96	230.42	675.27	352.89	614.90γ	20.50γ	35.54γ

* Increase for period equals comb-factor for period minus initial comb-factor minus capon comb-factor for period.
† Equivalent to Testosterone-propionate.

Two factors were important in the analysis of the comb-growth especially since we were interested in the quantitative effect of the exogenous androgen as well as the endogenous androgen secreted by the capon. First, there was some variability in the initial comb sizes in the different series. The chicks were operated at five days of age and some comb-growth occurred before experiments began on the 10th day. This was especially true of the unoperated males. At each period, therefore, the initial comb-factor was subtracted from that of the period in order to determine the net increment during the time interval. Second, there was a surprising growth of the combs of the capons. This growth, we have observed, exceeds that of the pullets and of ovariectomized females. This is an interesting endocrine problem in itself and we wish to treat this in more detail in a subsequent

study. Two possible explanations are suggested: (a) that the capon adrenal is stimulated to produce considerable androgen; or (b) that anterior pituitary hormone may act directly on the comb. The latter has been advanced as a hypothesis by Marlow 1950. Whatever the explanation there still remains the fact that considerable comb-growth occurs in capons. The comb-factors for each period, therefore, were further corrected by subtracting the capon comb-factor increment for each interval. Since both the initial size and the capon increment for each period were subtracted from



EXPLANATION OF GRAPH 1

GRAPH 1. Action of testosterone-propionate on comb growth as measured by comb-factors $\frac{(H \times L \text{ mm.})}{2}$ Each line represents the logarithm of comb-growth for series B through F for the period designated at the end of the line, plotted against the logarithm of T.P. dosage for series B through F respectively. Comb-growth for a period at each dosage level was determined by subtracting the initial comb-factor and the capon comb growth from the observed comb-factor for the period. The + signs indicate the comb-factors for uninjected normal male chicks. The lines were determined by calculating the regression equation for comb-factor against dosage. Series A, 0.5 gamma T.P. daily; B, 1.0 gamma T.P. daily; C, 5.0 gamma T.P. daily; D, 10.0 gamma T.P. daily; and F, 50.0 gamma T.P. daily.

the mean comb-factor of androgen treated capons and of unoperated males, it was felt that a fairly accurate estimate of androgen effect was possible both for a given period and for total growth.

Those who favor the target-organ hypothesis have challenged the data which demonstrate that gonadal hormones inhibit pituitary action. The basis for the challenge is the supposition that non-physiological doses of gonadal hormones were administered. This criticism cannot be discounted because very little is known concerning the amount of estrogen or androgen secreted by normal animals. Both the comb-factor and comb-weight data and pituitary weights furnish considerable information on this particular problem in our experiments.

It will be observed in Table 1 and in Graph 1 that the comb-factors for the male at 16, 21, and 26 days fall between the comb-factors for the capons which recieved 5 gamma T.P. and 10 gamma T.P. daily. Furthermore, at 16 days the male comb sizes were close to those of the capons given 5 gamma, but at 26 days the male comb sizes were very near those of the capons given 10 gamma. At 31, 35, and 40 days the male comb sizes exceeded those of capons which were given 10 gamma T.P. daily and assumed a position intermediate to those of capons which received 10 gamma and 50 gamma of T.P. daily. This same relationship exists for the comb weights and pituitary weights taken at 40 days at the termination of the experiments. It is possible to treat the data in a somewhat more elaborate statistical fashion by calculating the regression for the comb-factors on dosage and pituitary weight on dosage at 40 days.

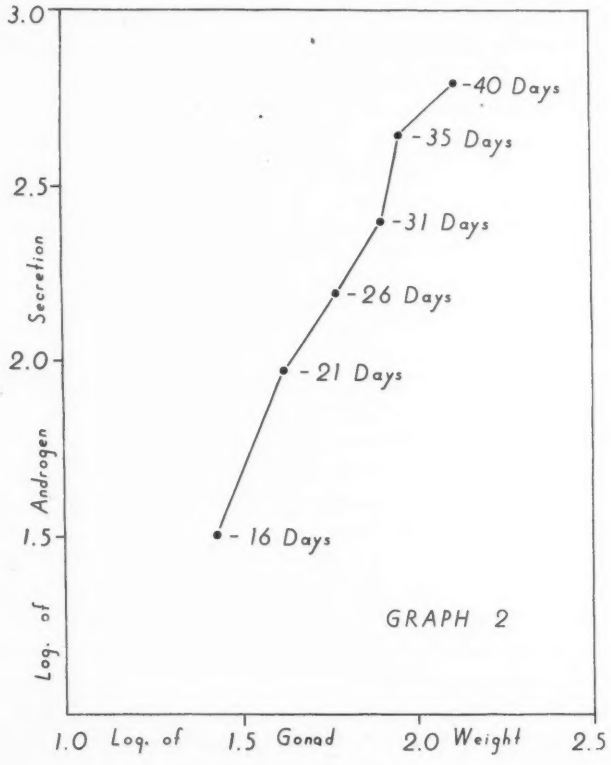
The determination of regression equations $\hat{y} = a + bx$ for each of the periods was made and since the comb-factors and comb-weights were known as well as pituitary weights it was possible to determine the relative amount of androgen secreted by the males for each period in terms of the equivalency to injected testosterone-propionate. The antrogen estimations are shown in Table 1 and as a footnote to Table 2.

TABLE 2. BODY WEIGHTS AND COMB WEIGHTS AT 40 DAYS. TREATMENT 10TH TO 39TH DAY

Series	No.	Body weight	Daily treatment	Final comb weight	Growth during period (final weight minus capon weight)
A ♂	11	374.8	0.1 cc. Sesame	317.3	—
B ♂	11	390.5	0.0005 mg. T.P.	363.2	45.9
C ♂	11	382.5	0.001 mg. T.P.	434.6	117.3
D ♂	11	372.1	0.005 mg. T.P.	598.2	280.9
E ♂	12	393.0	0.01 mg. T.P.	1,215.0	898.7
F ♂	10	397.8	0.05 mg. T.P.	3,880.0	3,562.7
G ♀	15	396.8	0.1 cc. Sesame	1,843.8	1,526.5
H ♀	14	383.4	0.1 cc. Sesame	232.8	—

Calculated equivalent of testosterone-propionate secreted by male during the 30 day period on the basis of comb weight increases was equal to 630.3 gamma.

The first observations which may be made relative to the amount of androgen secreted by the male on the basis of comb-factors is that from the 10th to 40th day. The androgen secreted was equivalent to 614.90 gamma of injected testosterone-propionate. It is very gratifying to note that on the basis of comb-weight the amount secreted was equivalent to 630.30 gamma of testosterone-propionate. The amount secreted on the basis of the pituitary data will be discussed later. The average daily secretion has little real significance as will be noted later but is, of course, in excess of 20 gamma equivalent per day. Greep and Jones estimated that the rats used in their study secreted between 10 gamma and 50 gamma per day. This is at least a significant physiological coincidence. Much more



EXPLANATION OF GRAPH 2

GRAPH. 2. The logarithms of the gonad weights for 523 chicks from 16 to 40 days grouped according to age are plotted against the logarithms of androgen secreted in terms of equivalent amounts of testosterone-propionate as calculated in Table 1 in the column titled Total Androgen Secreted. The age in days is shown at each of the points in the graph.

important than the average daily secretion, however, is the amount secreted during the different periods. These figures are shown in Table 1. The amount secreted from the 10th to 16th day was 31.74 gamma or 5.29 gamma per day, from the 10th to 21st day 94.56 gamma or 8.60 gamma daily, and from the 10th to 26th day 155.60 gamma or 9.72 gamma daily. There is a much greater relative increase after the 26th day, the amount being 250.80 gamma between the 10th and 31st days, 437.20 gamma between the 10th and 35th days, and 614.90 gamma between the 10th and 40th days.

The fact that the daily average secretion of 20.50 gamma for the period from 10 to 40 days is of little significance can be seen by reference to the right-hand column of Table 1. The amount of androgen secreted within the intervals differs greatly and shows an amazing increase after 31 days. The observation that the chick secreted considerable quantities of androgen early (before 10 days) and that there is a marked increase in the quantity at about 30 days post-hatching has been noted several times in our work; notably, Breneman (1945) and Breneman (1950). This is the first time, however, that it has been possible to determine quantitative differences. Previously the suggestion was made, Breneman (1945), that the amount of androgen being formed in the males appeared to have an inhibitory effect on the pituitary. Attention should be directed in this regard to the large amounts of androgen which is being released between the 31st and 35th day—46.60 gamma daily, and between the 35th and 40th day—35.54 gamma.

A total of 523 chicks was used to determine the gonad weights for the five day intervals from 10 to 40 days of age. The logarithm of androgen secreted by the gonads as shown in Table 1 was plotted against the logarithm of gonad weight and this is shown in Graph 2. The relationship is remarkably regular but one important variation is the small amount of gonad growth which occurs between 31 and 35 days of age. This is the area of the graph between the 4th and 5th points and it was noted above that during this period the estimated androgen secreted was 46.60 gamma. This suggests, therefore, that the inhibitory level of androgen would be about 40 gamma to 50 gamma. Fortunately, there is additional information on this problem furnished by the pituitary weight and assay data.

The possibility that non-physiological doses of the androgen could be detected by the effect on body weight has been considered by other workers and commented on by Greep and Jones. Our experimental animals were studied during their most rapid growth period as were the rats used by Greep and Jones. It was expected that if the androgens depressed growth it would be easy to detect this effect especially since it has been suggested that the effects are marked when the physiological dosage is reached and

exceeded. Reference to Table 2 will demonstrate, however, that there were no significant differences observed. The capons which were given only sesame oil were lighter than any other group except those given 0.005 mg. T.P. daily. The heavier groups, above 390 gms., show no correlation with dosage. As will be shown later the 0.05 mg. dosage was undoubtedly above the physiological level but the body weight in this series was the heaviest.

Pituitary weight and assay: It is especially important to analyze the data for the pituitary glands in these experiments because the dosage levels of the injected hormone were both below and above what we interpret to be the physiological level for normal cockerels. These data are summarized in Table 3 and a word of explanation should precede the dis-

TABLE 3. ANALYSIS OF PITUITARIES

Donors				Assay					
Series	No.	Daily treatment	Pit. average	Series	No.	Treatment	Gonad Range	Gonad Weight ave.	Chicks units* per pit.
A	10	♂ +Sesame oil	6.50 mg.	A′	16	4 mg. of A	7.8–21.3	13.25±0.99	6.29
B	9	♂ + 0.5 gamma T.P.	6.80 mg.	B′	15	4 mg. of B	7.9–21.2	12.25±1.09	5.71
C	9	♂ + 1.0 gamma T.P.	6.30 mg.	C′	14	4 mg. of C	8.7–17.9	12.53±0.81	5.51
D	10	♂ + 5.0 gamma T.P.	5.50 mg.	D′	13	4 mg. of D	6.3–18.0	11.47±1.06	4.07
E	10	♂ +10.0 gamma T.P.	5.10 mg.	E′	12	4 mg. of E	7.5–20.7	14.13±1.16	5.49
F	8	♂ +50.0 gamma T.P.	4.10 mg.	F′	8	4 mg. of F	3.7– 7.7	5.14±0.45	—
G	14	♂ +Sesame oil	4.60 mg.	G′	15	4 mg. of G	5.4–14.7	10.13±0.75	2.62
H	12	♀ +Sesame oil	4.10 mg.	H′	10	4 mg. of H	5.2–14.8	7.84±0.88	1.15
				I	18	Water	3.7– 8.0	5.63±0.29	—

* A net increase of 35% over the control represents one chick unit.

cussion of their significance. The test for total gonadotropic effect of the pituitaries using the baby chick is very effective and the action of the testosterone-propionate on pituitary weights is very consistent. The data reported by Greep and Jones in their Fig. 1, and Tables 6 and 8, which are comparable to our experimental procedures, are not as critical as our data for capons although similar conclusions can be reached.

First of all, there is a marked correlation between the decrease in average pituitary weight and the increase in the amount of androgen given the capons. The one exception is the increased pituitary weight in the series B which was given 0.5 gamma T.P. daily. Whether this is a real phenomenon or merely sampling variation we are not able to determine at present but we are inclined to believe that this is merely variation. Series F which received 50.0 gamma T.P. daily had a pituitary weight average considerably below that of the controls, Series G. It was noted in the discussion of androgen secretion by the males that the calculated amount released was between the 10 gamma and 50 gamma daily amounts. It was, therefore, particularly significant to observe that the cockerel pituitary weight was intermediate between that observed in series E and F, namely 4.60 mg. The females of series H were included here for both weight and assay

comparisons. The pullets have lighter pituitaries, a fact which was noted previously, Breneman 1946 and it happens that the glands weighed the same as those of series F.

There can be no doubt that the increase in the weight of the capon pituitary is inhibited by androgen injection and that this is closely correlated with the dosage employed. As an interesting comparison, the regression equation for the logarithm of pituitary weight on the logarithm of dose of testosterone-propionate was calculated after subtracting the average weight of the pituitary at 10 days which for 32 chicks was 1.6 mg. The estimation of the amount of androgen present in the male on this basis was 621.0 gamma. This is in very close agreement with the 614.9 gamma calculated for comb-factors and 630.3 gamma calculated for comb weights, and gives us considerable confidence that we have obtained a reasonably accurate estimate of androgen secretion in the cockerel.

The pituitary glands were assayed by injecting acetone-dried glands into baby chicks which were kept in shipping boxes ("Chick Pullmans") without food or water during the injection period. The testicular weights were used as a measure of gonadotropic potency. The average weight of the pituitary gland in each series was known and the same amount of glandular material, 4 mg. equivalent of fresh gland, was given. It is possible, therefore, to determine not only the per-milligram potency but also to estimate the total potency of the pituitary glands in the various series. This is a more critical analysis than was possible in the experiments of Greep and Jones in which varying amounts of pituitary gland were administered to their assay animals.

The assay data are summarized in the right half of Table 3. Eighteen animals, series I, were given only water injections and served as control chicks. The range of testis weights of 3.7 mg. to 8.0 mg. is consistent with that observed in other assays which we have run. The mean of 5.63 mg. is slightly higher than that usually encountered but probably reflects the fact that for this assay only strong active chicks were used. Any cockerels which appeared weak or inactive were discarded before injections began. The minimum and maximum testis weights in series A′ through E′ all exceeded the controls, as did also those of series G′ and H′ which were given control male and female pituitary glands. Only in series F′ was the range comparable to that of the water controls. It is important to analyse these results in more detail. Series A′ through E′ received pituitary material from the donor series A through E respectively, and the animals in these series were given from 0.5 gamma to 10 gamma of T.P. daily. The mean weight of the testes in these groups ranged from 11.45 mg. in series D′ to 14.13 mg. in series E′ and the differences are not statistically significant. Series F′, however, which was given pituitary gland from capons that re-

ceived 50.0 gamma daily of T.P. had a mean testis weight of 5.14 mg. slightly below that of the water injected controls. It is obvious that the 50 gamma dosage of T.P. completely inhibited the pituitary gland. It should be remembered that the pituitary weight was also greatly inhibited in series F, averaging only 4.10 mg. Likewise, in the normal chicks at 31 to 35 days of age where a calculated androgen secretion of 46.60 gamma daily was postulated testis growth appeared to be inhibited. The "non-physiological" dosage of T.P. would appear to be approximately 50 gamma daily.

It is interesting to compare series G′ and H′, which received normal male and female pituitaries, with the various capon series. The mean testis weight for the male, series G′, was 10.13 mg. and for the female, series H′ was 7.84 mg. The male pituitary is, therefore, more potent than that of the female but less potent than that of the capon. Furthermore, since the weight of the female glands by coincidence was the same as that observed for series F′ it will be seen that weight differences alone do not account for the differences in gonadotropic potency.

The pituitary weight and assay data furnish a very surprising picture when considered together because the weight of the capon pituitaries varies inversely with the dosage of the hormone administered but the potency per unit of weight is not decreased until the 50.0 gamma dosage level is reached. This fact led us to make some additional analyses of the experiments.

Since all assay chicks received the same amount of acetone-dried gland (4 mg. equivalent of fresh gland), and since the average pituitary weight in each series was known, it is possible to estimate the total activity of the pituitary gland in each series. This estimation was based on our previous observation that a net increase of 35% in testis weight was usually significant in this assay technique (Breneman 1945). We have called this a "chick unit." The total gonadotropic potency of the glands in terms of chick units is shown in the extreme right hand column of Table 3. There is a rather consistent decrease in the total activity of the pituitary as the dosage of androgen increases. The data for series D′ and E′ are the only ones deviating to any marked degree, but even these series are not far out of line, and the possibility that these increases may be real should be subjected to further study. These facts illustrate that the total gonadotropic content of the pituitary was lowered following the administration of even low dosages of androgen but that this decrease was due not to lower activity per unit of gland but to the decrease in the total amount of gland present.

The second important analysis of the action of the androgen is based on the cytological appearance of the glands. Dr. Fernandus Payne was kind enough to fix and stain two pituitaries from each of the series A

through H. The glands were given to him with only a serial number to identify them and he had no knowledge of the treament or sex of any of the animals from which the pituitaries were taken. His report on the cytological features of the glands was as follows: The pituitaries of the capon series A through E were indistinguishable insofar as cellular composition was concerned. All were composed almost exclusively of large active basophilic cells. Series G, the control males, had large numbers of active basophiles but much fewer than those present in the capon series. The pituitaries of series H, the females, had some active basophiles and many acidophiles, and differed in this respect from any of the other glands. The most striking differences, however, were in the pituitary glands of series F, from capons which were given 50.0 gamma of T.P. daily. These glands were notable because of a complete absence of any large active basophilic cells and the presence of numerous chromophobic cells. It is now generally accepted that the basophiles are responsible for the secretion of gonadotropins or at least of the follicle-stimulating-hormone (gametogenic), F.S.H. These independent observations of Dr. Payne on unknown material correlated beautifully with the variations in gonadotropic potency which we have summarized in Table 3.

The relation of the foregoing observations to the conflicting hypotheses of pituitary-gonad interaction may be best resolved by accepting a compromise viewpoint with somewhat greater emphasis on the inhibition of the pituitary by androgen. Certainly at a high dosage level, 50 gamma daily or above, the gonadotropic action of the pituitary is completely suppressed. At the low dosages of 0.5 gamma, 1.0 gamma, 5.0 gamma, and 10.0 gamma daily, which we believe to be within the range of "physiological dosages," the activity per unit of gland is not altered but the total weight of the gland is decreased. This means, of course, that in the overall physiological picture of the endocrine system the total amount of gonadotropin would be decreased. Since our observations were made on capons it is imperative that similar studies should be made on normal males, females, and ovariectomized females; such studies are in progress.

SUMMARY AND CONCLUSIONS

The growth of the capon comb as represented by either a comb-factor $\frac{(\text{Height} \times \text{Length mm.})}{2}$ or by comb weight resulted in a straight line when plotted as the logarithm of size against the logarithm of dosage of testosterone-propionate administered. Excellent comb growth was produced by as little as 0.5 gamma and 1.0 gamma of T.P. daily.

The determination of regression equations for the various periods during the experiment made it possible to estimate the amount of androgen se-

creted in cockerels at various ages from 10 days to 40 days post-hatching. The average daily amount secreted was equivalent to 20.50 gamma of testosterone-propionate. The secretion rate, however, was not uniform and a very marked increase occurred during the 30–40 day period. Considerable comb growth was evident in the control capons and this is recognized as affording a problem for future study.

The question of a "physiological dosage" of the androgen was considered. None of the dosages used had any significant effect on body weight. The pituitary weights of the capons given 50.0 gamma daily of T.P. had glands which were considerably smaller than those of the controls. The assay of the pituitaries demonstrated that per unit of weight the capon glands had more gonadotropic potency than those of the males and that the T.P. had no effect on this unit potency until the 50.0 gamma dosage was employed. This dosage, however, completely depressed the gonadotropic action of the gland and this indicates that there are two effects of injected androgen; first, on storage or secretion and second on gland weight.

A cytological study of the pituitaries made independently by Dr. Fernandus Payne showed that the variation in the gonadotropic activity of the glands could be correlated with the number and size of the basophilic cells present. The 50.0 gamma dosage completely suppressed the basophiles and the glands were largely composed of chromophobes, but the other dosages had no detectable cytological effect.

The fact that these data demonstrate inhibition of the gonadotropin content of the pituitary following injection of testosterone-propionate at both physiological and non-physiological dosage levels supports the "teeter-totter" hypothesis of gonad-pituitary interaction. It must be recognized, however, that at physiological dosage levels this inhibitory effect was produced in the capons solely as a result of decreased pituitary size and not by any suppression of the number and size of the basophilic cells. These observations seem to be more nearly in accord with the "target-organ" hypothesis.

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THE INHIBITION OF HAIR GROWTH BY ESTROGENS AS RELATED TO ADRENAL CORTICAL FUNCTION IN THE MALE RAT[1]

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THE systemic administration of large doses of estrogen over a period of several weeks suppresses the growth of hair in rats. Hair growth can be similarly inhibited by large doses of adrenal cortex extracts, 11-oxygenated steroids or of hypophyseal adrenocorticotropin (ACTH). It is known that large doses of estrogen cause stimulation of the adrenal cortex via the increased secretion of hypophyseal adrenocorticotropin. Hair growth is accelerated following adrenalectomy in the rat. Baker and Whitaker (1949) and Baker (In press) have reviewed the published data bearing on this problem and have demonstrated that whereas estrogen inhibits hair growth in non-adrenalectomized rats, it fails to inhibit hair growth in adrenally insufficient rats.

Baker and Whitaker (1949) have raised the question which is the subject of the present study, namely: Is the hair-growth inhibiting effect of estrogen mediated by an increase in the secretory activity of the adrenal cortex, or do the cortical hormones play a supporting role as Ingle (1950) has described for certain other metabolic adjustments? The data of this study show that estrogen has an effect upon hair growth in the rat which is not mediated by any change in the secretory activity of the adrenal cortices but which does require the presence of the cortical hormones for its manifestation.

METHODS

Male rats of the Sprague-Dawley strain having an initial weight of approximately 300 gms. were used in these experiments. The animals were adapted to the forced-feeding of a medium carbohydrate diet (Table 1) in the amount of 13 ml. each morning and late afternoon. The diet and procedures were modifications of those described by Reinecke, Ball and Samuels (1939).

Adrenalectomy was carried out under ether anesthesia according to Ingle and Griffith (1942). Asepsis was maintained in this operation. All of the rats were given 0.9 per cent sodium chloride solution to drink throughout the experiment. The adrenal cortex

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extract (ACE) represented 40 gms. of beef adrenal glands per ml. It was free from alcohol but contained 0.9 per cent sodium chloride. The ACE was added to the drinking water (saline), and the total volume was adjusted to meet the daily requirement of each animal. Alpha-estradiol (Ciba) was diluted with peanut oil so that each dose was given at 0.2 ml. daily by subcutaneous injection. The control animals received equal volumes of peanut oil.

The hair was clipped from the areas under observation at weekly intervals. An electric clipper with a 000 head was used. In the first experiments, the pattern of hair growth on the whole body was sketched each week prior to clipping. In the last 3 experiments, the growth of hair was studied on one side of the body in an area bounded by the mid-dorsal and mid-ventral lines and the anterior and posterior extremities. In addition to

TABLE 1. MEDIUM CARBOHYDRATE DIET

Constituent	Grams
Cellu flour (Chicago Dietetic Supply)	120
Osborne & Mendel salt mixture	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Mazola oil plus 100 mg. Vitamin K	10
Mazola oil	190
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of cc.	2,000

the sketching of the patterns of hair growth, the clipped hair was carefully collected and weighed. At necropsy, a strip of skin, extending from the mid-ventral to the mid-dorsal line was prepared for histological study. Sections of these strips of skin were stained with iron hematoxylin and Masson, hematoxylin, and eosin and the Weigert stain for elastic fibers.

EXPERIMENTS AND RESULTS

In experiment 1, one rat of each of 6 pairs was given 20 μg. of estradiol daily for 9 weeks. At the end of the 5th week, 2 pairs of rats were subjected to sham adrenalectomy; 2 pairs of rats were adrenalectomized and were maintained without ACE; and 2 pairs were adrenalectomized and given 5 ml. of ACE daily. The estrogen-treated rats showed partial suppression of hair growth by the end of 4 weeks. The injection of oil in the control animals was without effect upon the growth of hair. Sham-adrenalectomy did not modify the inhibitory effect of estrogen upon hair-growth. Following adrenalectomy without subsequent treatment with ACE, the estrogen failed to maintain the inhibition of hair-growth. Regrowth of hair was evident 3 weeks following adrenalectomy. In contrast, treatment of adrenalectomized rats with 5 ml. of ACE daily sustained the inhibition of hair growth effected by the estrogen but did not prevent the growth of hair in the adrenalectomized animals which were given control injections (oil).

Experiment 2 was identical with experiment 1 except that the daily dose of estrogen was 40 μg. daily. The results were relatively the same. The inhibition of hair growth by this dose of estrogen was almost complete yet the inhibition was not sustained in the adrenalectomized rat unless the animal was treated with ACE (5 ml. daily). The results are illustrated by the drawings of hair growth patterns in Figure 1.

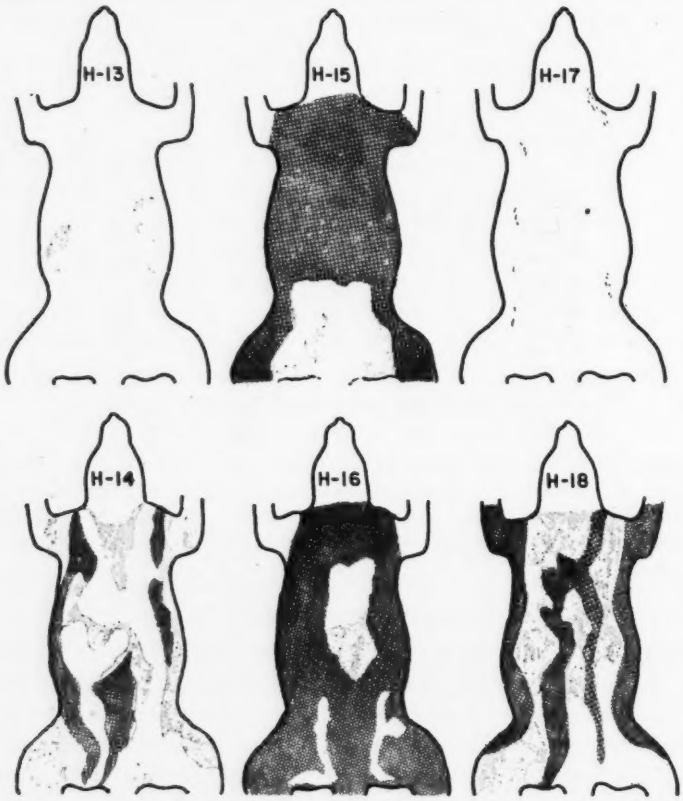

FIG. 1. Drawings of hair pattern grown during the 4th week following operation. The thickness of growth is represented by the intensity of shading. H-13, sham adrenalectomy, estrogen; H-14, sham adrenalectomy, control; H-15, adrenally insufficient, estrogen; H-16, adrenally insufficient, control; H-17 ACE-treated adrenalectomized, estrogen; H-18, ACE-treated, control.

In experiment 3, one rat of each of 6 pairs was given 40 μg. of estrogen daily for 14 weeks. At the end of 4 weeks, 2 pairs of rats were subjected to sham-adrenalectomy, 2 pairs of rats were adrenalectomized and kept

without treatment with ACE, and 2 pairs were adrenalectomized and treated with 10 ml. of ACE daily. The general results were identical with those of experiments 1 and 2. Estrogen inhibited hair-growth in the presence of the adrenal glands, failed to sustain inhibition of hair growth in adrenally insufficient animals, but did continue to inhibit hair growth in adrenalectomized rats treated with ACE.

Experiment 4 was identical with experiment 3 except that the animals

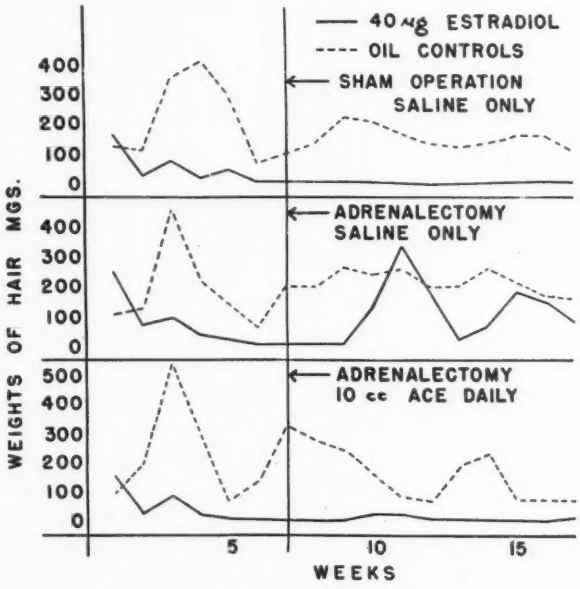

FIG. 2. The effect of estrogen upon hair growth on one-half of body as related to adrenal cortex function. Averages of 2 rats per group.

were operated at the end of the 7th week and the observations were continued for 10 weeks postoperatively. The data on weights of hair which was clipped at weekly intervals are summarized in Figure 2.

Experiment 5 involved 12 adrenalectomized rats. The animals were treated with saline for two weeks following operation. Thereafter, each rat received 8 ml. of ACE daily for a period of 9 weeks. One rat of each pair was given 40 μg. of estrogen daily. The data on weights of hair are summarized in Figure 3. The administration of estrogen caused suppression of hair growth which became evident by the end of 4 weeks. The control animals which received the same amounts of ACE showed some temporary decrease in hair growth.

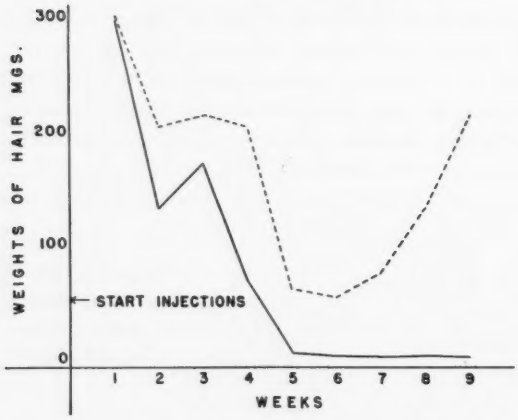

FIG. 3. The effect of estrogen upon hair growth on one-half of body of adrenalectomized rats treated with 8 ml. of ACE daily. Averages of 6 rats per group.———— estrogen, ----------control.

MICROSCOPIC OBSERVATIONS

In the skin of non-adrenalectomized rats treated with estrogen, there were practically no growing hair bulbs. Almost all of the follicles were inactive, extending not more than about one-third of the distance through the dermis (Figs. 4a and 4b). In contrast, if adrenalectomy had been performed, continued growth of hair in spite of estrogen treatment was confirmed by the presence of many hair bulbs extending into the subcutaneous fat. When ACE was administered to adrenalectomized rats concurrently with estrogen, the hair follicles were brought to a morphological state comparable with that induced by estrogen alone in non-operated animals (Fig. 4d). In some of the rats which had been adrenalectomized one month prior to the initiation of the injections, this inactivation of the hair apparatus had been so complete that epithelial buds were almost totally absent.

Some thinning of the epidermis occurred in non-operated animals treated with estrogen which confirms previously published reports (Hooker and Pfeiffer, 1943; Baker and Whitaker, 1949). Estrogen did not elicit this effect after adrenalectomy with or without the concurrent administration of ACE. The dermis was reduced in thickness by estrogen in both non-operated and adrenalectomized rats. However, the microscopic structure of the dermis was not modified except that the tissue became more compact and the collagenous fibers somewhat smaller. No changes were observed in the dermal elastic fibers.

In non-operated rats, estrogen induced only a slight loss of fat from the

panniculus adiposus which stands in contrast to the complete depletion of this fat which occurred in rats injected with ACTH (Baker, Ingle, Li and Evans, 1948). Failure of estrogen and ACTH to bring about similar effects may have been due to a difference either in physiological action or in dosage. Likewise, in adrenalectomized rats, subcutaneous fat remained after treatment with estrogen and ACE separately. Failure of extract to

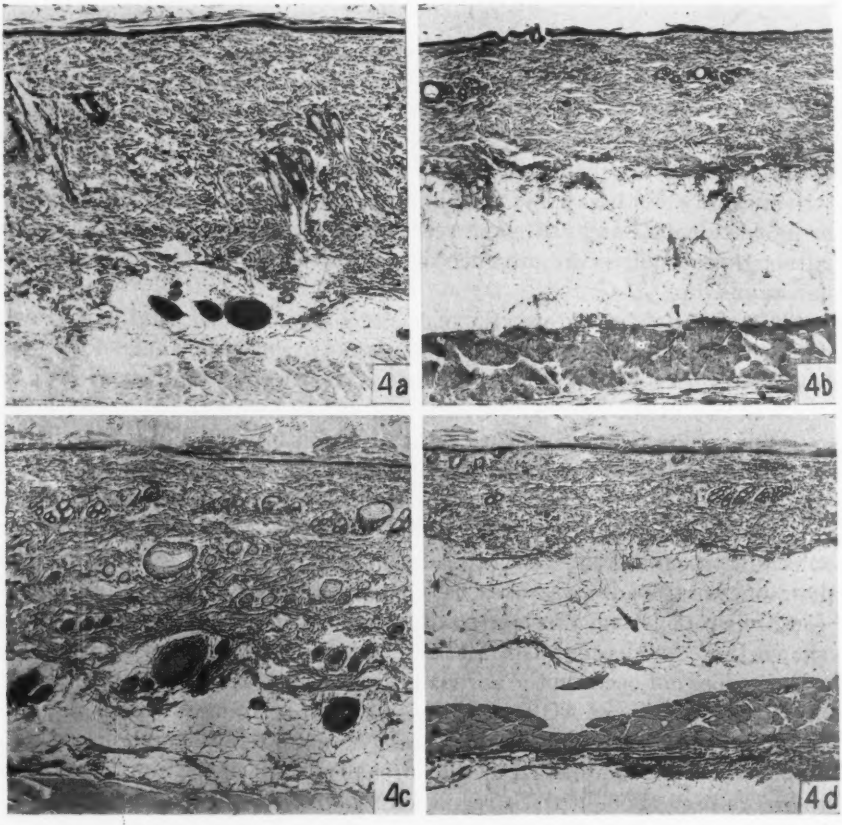

FIG. 4. Photomicrographs of skin. H. and E. ×120. 4a. Rat H-38. Sham adrenalectomy control for H-43. The bulbs of actively growing hair follicles are present in the panniculus adiposus. 4b. Rat H-43. Sham adrenalectomy. 40 μg. of estradiol daily for 7 weeks before and for 10 weeks after operation. The epidermis and dermis are thinner. Hair is not growing. The panniculus adiposus is thick. 4c. Rat H-56. Adrenalectomy 2 weeks prior to the experiment. 8 ml. of Ace daily for 9 weeks. No estrogen. Compared to 4a, no significant changes have been induced. 4d. Rat H-57. Treatment as for H-56 except that 40 μg. of estradiol was administered daily with ACE. Hair is not growing and the panniculus adiposus is thick. The dermis is thinner than in 4c.

reduce significantly the thickness of the panniculus adiopsus was due to insufficient dosage, since with more intense treatment, the fat may be removed completely from this region (Baker and Ingle, unpublished observations). When estrogen and ACE were administered together, the amount of fat in this region frequently was greater than under any of the other conditions of treatment in this experiment.

DISCUSSION

These data confirm the observations of Baker and Whitaker (1949) in showing that the characteristic inhibition of hair growth by large doses of estrogen does not occur in the adrenally insufficient rat. The hypothesis that an increase in the secretory activity of the adrenal cortex mediates all of the effect of estrogen upon hair growth is not supported by the data of this study. Estrogen has an inhibitory effect upon hair growth which is independent of any change in secretory activity of the adrenal cortices although it requires the presence of cortical hormones for its full manifestation.

The hair of the rat grows in cycles and the amount of total hair grown per unit time is highly irregular. The data on weights of hair (Figures 2 and 3) suggest, but do not prove, that estrogen inhibited hair growth to some extent in the adrenally insufficient rat and that the administration of ACE without estrogen caused some inhibition. However, the results of each experiment were consistent in showing that estrogen caused an almost complete blocking of hair growth in non-adrenalectomized and in ACE-treated adrenalectomized rats but failed to have its full inhibitory effect in the adrenally insufficient rat. Ingle (1943) has shown that the hormones of the adrenal cortex have a similar relationship to the diabetogenic effect of estrogen in the rat. Ingle, Ward and Kuizenga (1947) have shown that the characteristic rise in urinary nitrogen following fractures of bone does not occur in the adrenally insufficient rat but is supported by the administration of ACE which was uniform in amount during the pre-fracture and the post-fracture periods. There are many metabolic adjustments which are suppressed in the adrenally insufficient animal and have therefore been thought to be regulated by changes in the secretory activity of the adrenal cortices. These metabolic responses must be studied in adrenalectomized animals given a steady intake of adrenal cortical hormones before it can be decided whether the cortical hormones play a regulatory role based upon changes in the quantitative secretion of the hormones or whether the role of the hormones should be characterized as supporting.

The appearance of the adrenalectomized rats treated with estrogen deserves some comment. Most investigators, including ourselves, have

found large doses of estrogen to be toxic to adrenally insufficient rats. In these experiments, the rats were force-fed, given saline to drink and were adapted to the estrogen prior to adrenalectomy. Under these conditions, the overt appearance and behavior of these animals suggested that the administration of estrogen may have had a favorable effect upon these animals. At least estrogen did not preceipitate signs of adrenal cortical insufficiency. A systematic study of the effect of estrogen treatment prior to and following adrenalectomy is indicated.

SUMMARY

Male rats of the Sprague-Dawley strain were force fed a medium carbohydrate diet during a study of the effect of large doses of estrogen (20 to 40 μg. day) on hair growth. The following experimental conditions were represented: (1) sham adrenalectomy, no estrogen; (2) sham adrenalectomy, estrogen; (3) adrenalectomy, no adrenal cortex extract (ACE) and no estrogen; (4) adrenalectomy, no ACE, estrogen; (5) adrenalectomy treated with ACE (5 to 10 ml. day) no estrogen; and (6) adrenalectomy treated with ACE, estrogen. Two rats were studied under each of these conditions in each of 4 separate experiments. Estrogen inhibited hair growth in non-adrenalectomized rats and in adrenalectomized rats treated with ACE but failed to sustain inhibition of hair growth in adrenally insufficient rats. In a 5th experiment, 12 adrenalectomized rats were treated with ACE for a period of 9 weeks. Six of the rats received estrogen and 6 did not. Estrogen almost completely inhibited hair growth whereas hair continued to grow in the ACE-treated rats without estrogen. Microscopic observations of the skin showed that estrogen suppressed the activity of the hair follicle in non-adrenalectomized rats and in adrenalectomized rats treated with ACE but did not have this effect in the adrenally insufficient rats.

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A CYTOCHEMICAL STUDY OF LIPIDS IN SOWS' OVARIES DURING THE ESTROUS CYCLE[1,2]

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THE present study concerns the lipids in sows' ovaries, investigated by a group of cytochemical tests which characterizes steroid-producing cells. Earlier, Dempsey and Bassett (1943) and MacKay and Robinson (1947) employed similar tests to investigate the ovaries of rats and of humans. Sows' ovaries are readily available, and Corner's (1921) measurements of the relative sizes of their follicles and corpora lutea during the several phases of the estrous cycle permit one to date the ovaries without recourse to vaginal smears. The sow, like the human, has a long reproductive cycle (21 days) with a typical luteal phase, and its ovary also contains relatively little interstitial tissue. By the study of a suitably large number of specimens from different phases of the cycle, it was hoped that the sites of hormone production as well as the periods of most active secretion could be ascertained.

MATERIALS AND METHODS

Determination of the stage of the cycle. The ovaries from 25 sows were studied.[3] These were dated in the estrous cycle according to the method of Corner (1921) by measuring the diameters of the follicles and corpora lutea protruding from their surfaces. None of the ovaries selected belonged to uteri which were visibly pregnant. For metestrus (first 3 days after ovulation), 4 ovaries were selected with follicles less than 5 mm. in diameter and corpora lutea of 6 to 7 mm. in diameter, containing visible blood clots. For diestrus (4 to 17 days after ovulation), 6 glands were chosen with follicles of 5 mm. and corpora lutea of 9 or 10 mm., with a pink tinge. For proestrus-to-estrus (18th day to ovulation), 8 ovaries were selected with clear, translucent follicles measuring 8 to 10 mm. and corpora lutea of about 6 or 7 mm., with a yellowish tinge. On the basis of subsequent histological observations the ovaries could be classified more precisely. The remaining 7 ovaries contained one or more abnormal structures.

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[2] This work was aided by grants from the American Cancer Society: (1) on the recommendation of the Committee on Growth of the National Research Council, and (2) from an Institutional Grant to Harvard University.

[3] Kindly supplied by John F. Squire and Co., Cambridge, Mass., and Armour and Co., Chicago, Illinois.

Histological procedures. The ovaries were bisected. For routine histological study, one-half of each was fixed in Bouin's fluid for 24 hours. For the study of lipids, the other half was fixed in a solution of 10% neutralized formalin for a week or longer. The blocks were immersed in the fixing solutions within 20 to 30 minutes after the animal was killed.

The blocks fixed in Bouin's fluid were dehydrated in alcohol and chloroform and then embedded in paraffin. Sections were cut at 10μ and stained with Harris' hematoxylin and eosin.

The formalin-fixed specimens were first washed in running tap water for an hour, then sectioned at 20 μ on a freezing microtome. Most of them were cut without the use of any embedding medium, but the 8 specimens with large follicles were infiltrated with an aqueous solution of gum acacia and sugar at room temperature for 24 hours before cutting. This embedding mixture consisted of equal parts of a 75% solution of gum acacia and a saturated solution of sucrose. One section from each ovary was stained for lipids in a 1% solution of sudan black B in 70% alcohol for 4 minutes. Other sections were stained for carbonyl groups with Schiff's reagent without the use of mercuric chloride or by the Ashbel-Seligman (1949) procedure. The above sections were mounted in glycerin jelly. An additional section from each ovary was mounted unstained and studied under the polarizing and fluorescence microscopes. Control sections were extracted for 1 hour with acetone at room temperature and then prepared by each of the methods listed above.

OBSERVATIONS

The ovarian follicles. Primordial follicles, lacking an antrum, exhibit lipid only in the ovum (Figs. 1 and 2). At this and subsequent stages in follicular development, the droplets in the ovum dissolve in acetone, react to carbonyl tests, and contain birefringent crystals (Fig. 3).

As soon as the follicles become vesicular, they acquire lipid in the basal cells of the granulosa as well as in the cells of the developing theca interna (Fig. 4; also 24). The droplets in the granulosa are occasionally birefringent (Figs. 5 and 8) but fail to react with carbonyl reagents and are not fluorescent. They are entirely soluble in acetone. During the preovulatory swelling of the follicles, in proestrus-to-estrus, the droplets of the basal cells diminish in number, whereas a diffuse cytoplasmic staining of all of the granulosa cells with sudan black and the carbonyl reagents emerges (Figs. 6 and 7).

The lipid droplets which develop in the cells of the theca interna are brightly birefringent throughout the life of the follicles (Figs. 3, 5 and 8). During proestrus, as the thecal cells enlarge and begin to indent the granulosa, the droplets increase in number (Fig. 6) and acquire a weak carbonyl reaction (Fig. 7) and a yellowish fluorescence. Their birefringent crystals appear finer and more numerous (Fig. 8).

The corpora lutea. During the first 3 days after ovulation (metestrus), some of the large theca interna cells remain at the periphery of the developing corpora lutea, while others invade the granulosa with the sprouting

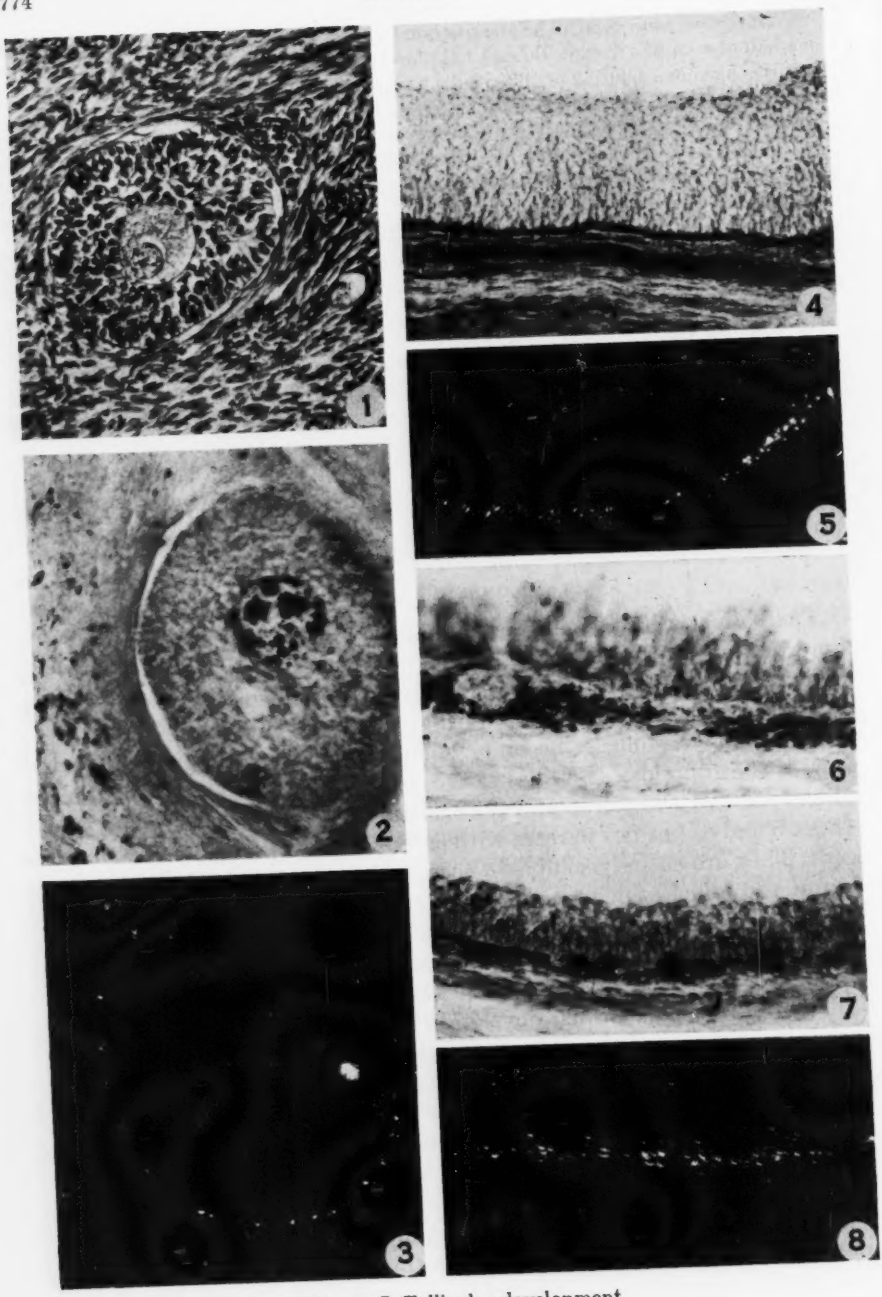

PLATE I. Follicular development.

blood vessels. The cells of granulosa origin begin to hypertrophy. All of the lutein cells display numerous large lipid droplets; the droplets are most crowded, however, in the theca lutein cells and in those granulosa lutein cells which lie adjacent to the central coagulum (Figs. 9 and 10). Many of the droplets in both types of lutein cells react with carbonyl reagents (Fig. 11) and display coarse birefringent crystals. The droplets of the theca cells display a yellowish fluorescence. All of the reactions mentioned are prevented by prior extraction with acetone. A diffuse yellowish-white fluorescence of the innermost granulosa lutein cells remains unaffected by acetone treatment.

During early diestrus (from 4 to 7 days after ovulation), the granulosa lutein cells continue to hypertrophy until the corpora lutea attain their definitive size and structure (Fig. 12). During this stage and throughout the peak of diestrus (8 to 14 days), the over-all concentration of lipid is lower than during metestrus. The droplets remain numerous in the theca lutein cells, so that these are readily distinguishable from the lighter granu-

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FIG. 1. Hematoxylin and eosin, photographed with yellow and green filters, ×180. One large primary follicle, just prior to antrum formation. A theca interna has not yet differentiated.

FIG. 2. Sudan black B, photographed with a blue filter, ×180. A large primary follicle just prior to antrum formation, without a theca interna. Dense accumulations of fatty material occur normally in the ovum. No lipid is seen in the granulosa cells.

FIG. 3. Birefringence, ×75. A small vesicular follicle showing intense birefringence in the ovum, and a sparse scattering of birefringent crystals in the cells of the theca interna.

FIG. 4. Sudan black B, photographed with a blue filter, ×180, The wall of a vesicular follicle of diestrus, showing sudanophilic lipids in the basal layer of the granulosa as well as in the cells of the theca interna.

FIG. 5. Birefringence, ×75. The wall of a normal vesicular follicle of diestrus. Medium-sized birefringent crystals occur in the theca interna. A few are apparent in the basal layer of the granulosa.

FIG. 6. Sudan black B, photographed with a blue filter, ×180. The wall of a follicle undergoing preovulatory enlargement (8 mm. in diameter). Blood vessels in the theca interna are seen to be indenting the granulosa. Numerous sudanophilic droplets are present in the theca interna. The discrete layer of lipid in the basal cells of the granulosa has become dispersed. The background cytoplasmic sudanophilia of the granulosa cells has increased (cf. Fig. 4).

FIG. 7. Ashbel-Seligman reaction, photographed with a red filter, ×180. The wall of a proestrous follicle showing occasional small droplets in the theca interna, which react with carbonyl reagents. The prominent dark-staining areas in the theca interna are accumulations of red blood cells.

FIG. 8. Birefringence, ×75. The wall of an enlarging follicle, showing numerous fine birefringent crystals in the theca interna. Also evident are some very fine crystals in the granulosa. This picture of proestrus reflects an increase in number but a decrease in the size of the anisotropic crystals (cf. Fig. 5).

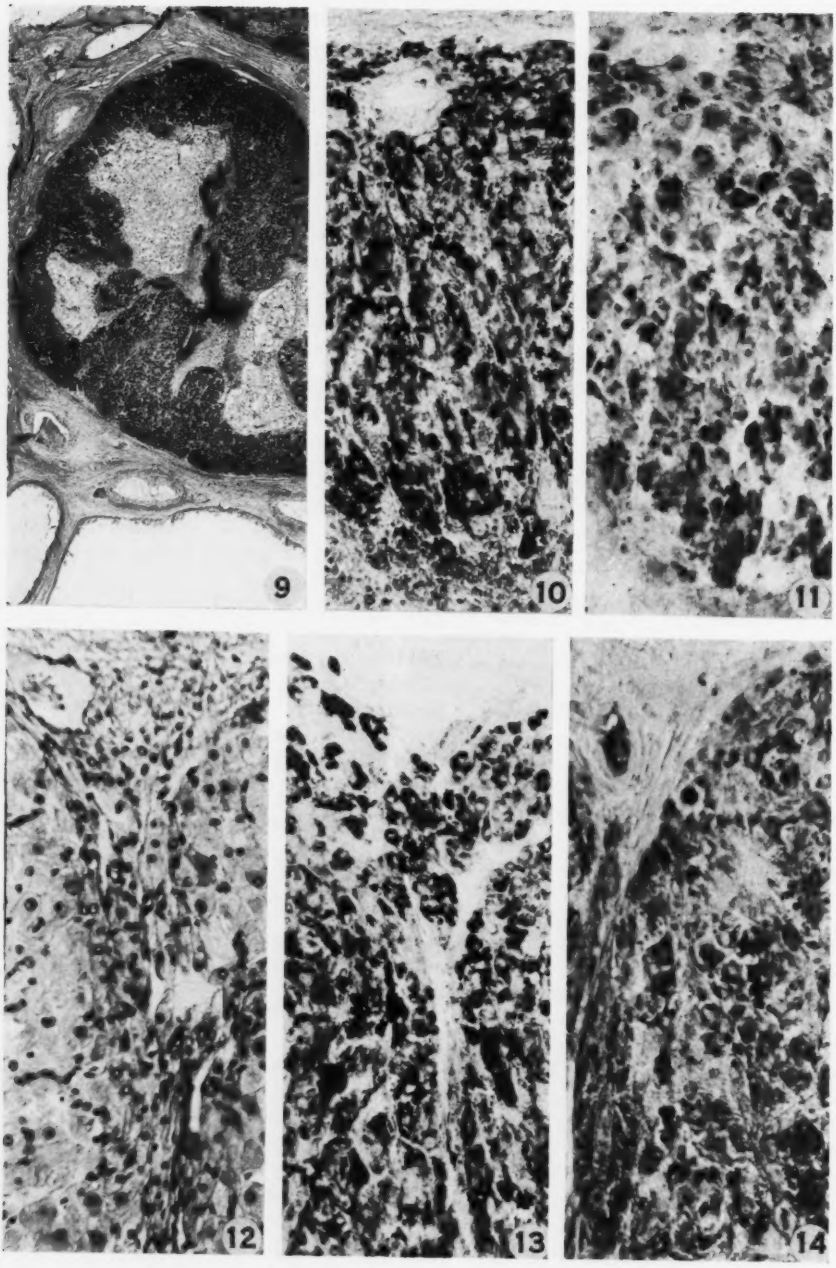

PLATE II. Corpora lutea of metestrus and diestrus

losa cells (Fig. 13). The droplets react with carbonyl reagents (Fig. 14) and contain numerous fine birefringent crystals (Fig. 15). Those in the theca cells also fluoresce. Throughout this period the parenchymal cells are also characterized by a diffuse background staining with sudan black and the carbonyl reagents. Unlike the droplets, which are entirely removed by acetone extraction, this stainable material is unaffected by the solvent.

During the period of late diestrus and of proestrus-to-estrus (15 days to ovulation), the corpus luteum begins to degenerate. The structure becomes hyperemic (Fig. 18); the central cells in particular become pycnotic; and a central scar makes its appearance. Its over-all sudanophilia increases. The birefringent crystals become larger but less numerous than heretofore (Fig. 16). Most of the droplets, except those occupying the peripheral theca lutein cells, lose their reactivity to carbonyl reagents (Fig. 17). The general cytoplasmic reaction with sudan black and carbonyl reagents declines. Many of the droplets in the more pycnotic cells are unextractable with acetone.

The age of older and more atrophied corpora lutea has been judged from the histological information presented by Corner (1915, 1921). Corpora of previous estrous cycles continue to shrink and are entirely unrecognizable after 4 generations. This shrinkage involves progressive pycnosis of the lutein cells and replacement of the parenchymal tissue by a connective tissue scar. During the second generation, the corpus becomes

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FIG. 9. Sudan black B, photographed with a blue filter, ×9. A newly formed corpus luteum, showing the folded appearance of its wall at this time. Centrally, the remnant of the extravasated blood persists. All of the cells of the corpus are sudanophilic. Below the corpus are several vesicular follicles which appear atretic.

FIG. 10. Sudan black B, photographed with a blue filter, ×180. The wall of a corpus luteum in metestrus. Sudanophilic droplets occur in both the theca and granulosa lutein cells. Most occur in the theca cells at the periphery and along the vascular channels and in the granulosa cells at the border of the central clot.

FIG. 11. Ashbel-Seligman reaction, photographed with a red filter, ×180. Wall of a corpus luteum in metestrus, demonstrating that some of the lipid droplets (Fig. 10) are reactive to carbonyl tests.

FIG. 12. Hematoxylin and eosin, photographed with yellow and green filters, ×180. Edge of a fully formed corpus luteum of diestrus showing large granulosa lutein cells, with the smaller theca lutein cells at the periphery and along the radial septum. Large sinusoidal blood vessels may be seen among the theca cells, and smaller ones penetrate between the strands of granulosa lutein cells.

FIG. 13. Sudan black B, photographed with a blue filter, ×180. Edge of a corpus luteum like that shown in Fig. 12. Crowded sudanophilic droplets occur in the theca lutein cells; smaller and sparser droplets characterize the granulosa cells.

FIG. 14. Ashbel-Seligman reaction, photographed with a red filter, ×180. The edge of a fully formed corpus luteum at the peak of diestrus, showing scant, small, carbonyl-reactive droplets in the theca and granulosa lutein cells.

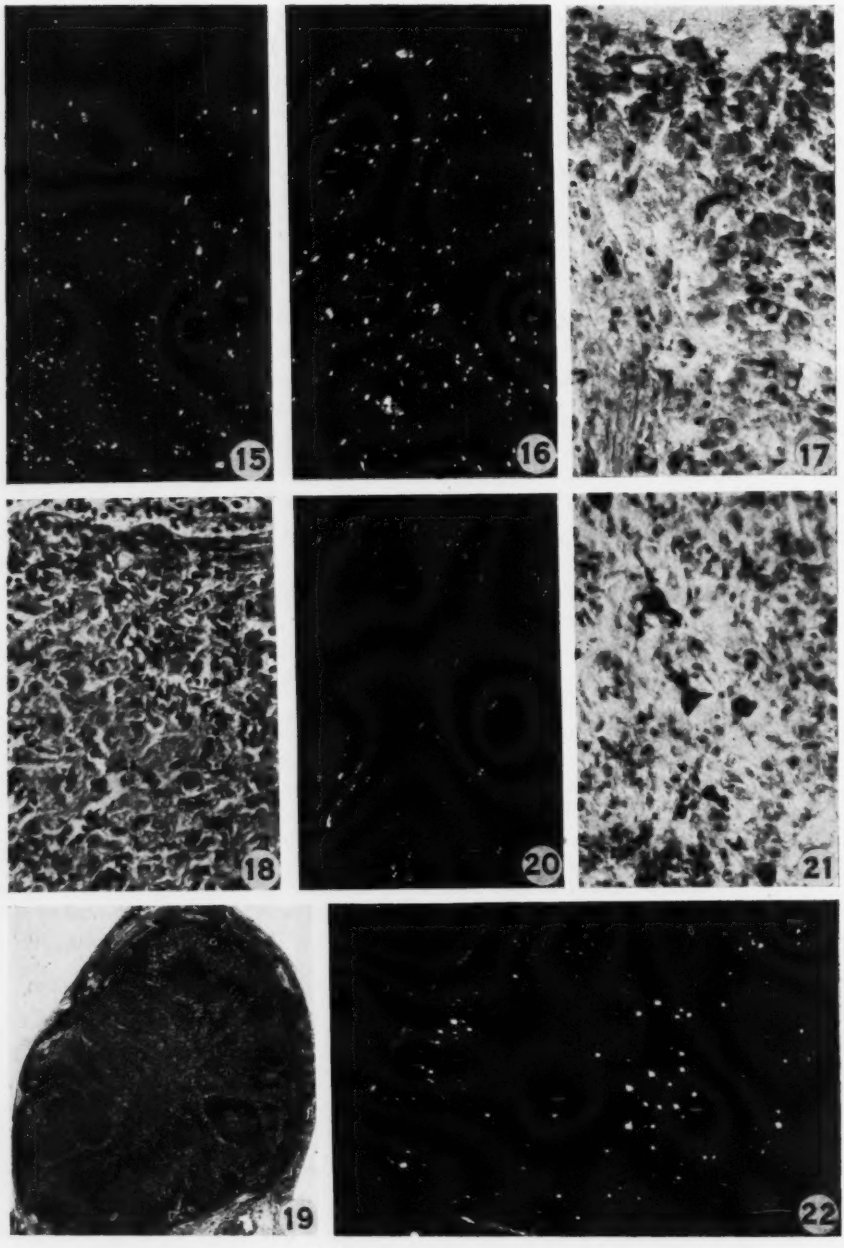

PLATE III. Corpora lutea of diestrus and proestrus and of earlier cycles

exceedingly fatty. The lipid occurs in the residual lutein cells and also apparently in connective tissue cells (Fig. 19). Most of this material is unaffected by acetone and is birefringent (Fig. 20), fluorescent, and intensely reactive to carbonyl tests. The quantity of lipid gradually declines and becomes progressively more insoluble as the structure ages (Figs. 21 and 22).

Follicular atresia. Follicles are seen to undergo atresia at all stages of their development as well as at all periods of the estrous cycle (Figs. 23 to 28). Some differences have been observed between those becoming atretic at the time of ovulation and those during diestrus. In those which become atretic during diestrus, the granulosa cells become fatty (Figs. 23, 24, 25 and 27). This fat evinces no reaction other than sudanophilia. The theca interna cells sometimes disintegrate (Fig. 27), sometimes become abnormally fatty (Fig. 25). In the latter case, much of the lipid is unaffected by acetone extraction.

When atresia occurs just before or just after ovulation, the condition more nearly resembles the picture in ovulating follicles. The theca interna indents the granulosa (Fig. 26) and its droplets become small and their

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FIG. 15. Birefringence, ×75. The wall of a mature corpus luteum of diestrus. Fine birefringent crystals are scattered abundantly throughout.

FIG. 16. Birefringence, ×75. Corpus luteum of late diestrus. The birefringent crystals are larger than before. Some of this material was found to be insoluble in acetone.

FIG. 17. Ashbel-Seligman reaction, photographed with a red filter, ×180. The edge of a corpus luteum in an intermediate stage of retrogression (proestrus). A great accumulation of large reactive droplets appears at the periphery, in the theca lutein cells. Few droplets are found in the granulosa lutein cells, which at this time are undergoing rapid degeneration.

FIG. 18. Hematoxylin and eosin, photographed with yellow and green filters, ×180. The edge of a corpus luteum in proestrus, showing marked engorgement. The lutein cells are reduced in size (cf. Fig. 12) and appear pycnotic.

FIG. 19. Sudan black B, photographed with a blue filter, ×9. A retrogressing corpus luteum, probably second generation. The entire structure is intensely sudanophilic; most of this material was unaffected by acetone extraction.

FIG. 20. Birefringence, ×75. Edge of corpus luteum shown in Fig. 19. The birefringent crystals shown here were largely unaffected by extraction with acetone.

FIG. 21. Ashbel-Seligman reaction, photographed with a red filter, ×180. The center of an old corpus luteum, probably third generation. The entire structure is undergoing degeneration. Most of the lutein cells have disappeared and been replaced by connective tissue. In the center of the fibrotic area, occasional cells contain large globules of carbonyl reactive material. This was entirely unaffected by acetone.

FIG. 22. Birefringence, ×75. Large birefringent crystals characterizing the old corpus luteum, probably third generation. These crystals are distributed in clumps and most are unaffected by acetone extraction. In later generations all of the lipids disappear.

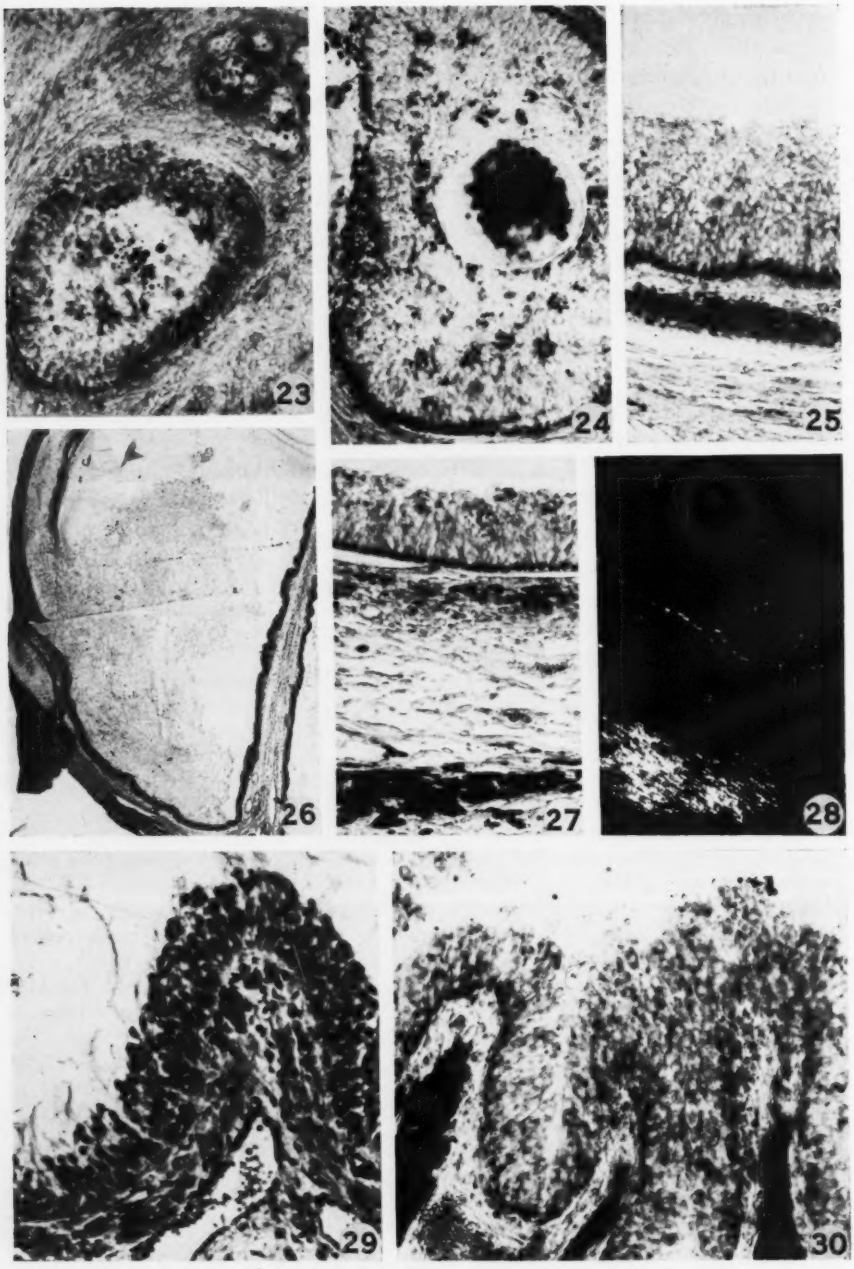

PLATE IV. Follicular atresia, cystic follicles, and interstitial tissue

birefringent particles fine (Fig. 28). Small lipid droplets develop in all of the granulosa cells.

In several of the ovaries studied, large cystic follicles occur. Most of these display greater than normal accumulations of "steroidal" lipid in the enlarged theca interna, as well as smaller reactive droplets in the granulosa (Figs. 29 and 30). Much of the thecal material remains after extraction with acetone, however.

Interstitial tissue. Many of the ovaries reveal small clusters of epithelioid cells in the stroma of the cortex which resemble the interstitial cells so abundant in rodents' ovaries. They present large accumulations of sudanophilic droplets (Fig. 27), most of which are insoluble in acetone. The drop-

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FIG. 23. Sudan black B, photographed with a blue filter, ×180. A young follicle, prior to antrum formation, undergoing atresia. The ovum has begun to fragment. The granulosa cells contain sudanophilic droplets. In the upper righthand corner is a cluster of primordial follicles undergoing atresia.

FIG. 24. Sudan black B, photographed with a blue filter, ×180. A small vesicular follicle undergoing atresia. The ovum contains abnormal amounts of lipid; fatty degeneration of the granulosa is occurring. The sudanophilia of the basal layer of the granulosa and of the theca interna is normal.

FIG. 25. Sudan black B, photographed with a blue filter, ×180. Wall of a follicle of diestrus undergoing early atresia. Large accumulations of sudanophilic material appear in the theca interna. The sudanophilia of the basal layer of the granulosa is not so discrete as in Fig. 4, and small fatty droplets appear scattered throughout the granulosa.

FIG. 26. Hematoxylin and eosin, photographed with yellow and green filters, ×9. A large follicle of proestrus undergoing incipient atresia, showing invasion of the theca interna into the granulosa (see Fig. 28).

FIG. 27. Sudan black B, photographed with a blue filter, ×180. Early atresia of a large vesicular follicle of diestrus. The granulosa cells are undergoing fatty degeneration. The basal layer of the granulosa displays its characteristic sudanophilia (cf. Figs. 4 and 25). The theca interna is discontinuous and contains only rare, large lipid droplets. Below the follicle lies a strand of interstitial tissue, displaying massive accumulations of lipid. Some of the latter material was acetone-insoluble.

FIG. 28. Birefringence, ×75. Edge of an atretic follicle resembling that shown in Fig. 26. The birefringence of the theca interna is fine, as is typical of rapid secretion in proestrus and estrus (cf. Fig. 8). Below, lies a mass of interstitial tissue with massive accumulations of coarse birefringent crystals. Most of the latter material was insoluble in acetone.

FIG. 29. Hematoxylin and eosin, photographed with yellow and green filters, ×180. The wall of a cystic follicle showing hypertrophy of the granulosa cells and of the cells of the theca interna. The theca interna and its associated blood vessels are invading the granulosa.

FIG. 30. Sudan black B, photographed with a blue filter, ×180. A cystic follicle similar to that shown in Fig. 29. Dense accumulations of sudanophilic material occur in the theca interna. There is only a remnant of lipid in the basal layer of the granulosa, and small, scattered lipid droplets appear in all of the enlarged granulosa cells.

lets react with carbonyl reagents, display birefringent crystals (Fig. 28) and emit a yellowish fluorescence.

DISCUSSION

The purpose of this cytochemical study of lipids in sows' ovaries has been (1) to define the sites of hormone production, and (2) to determine if possible the periods of active secretion.

(1) Cytochemical study of the organs of a variety of mammals has led to the conclusion that sudanophilia, together with a carbonyl reaction, birefringence, yellowish-green fluorescence and acetone solubility, characterizes the cytoplasmic droplets of only the steroid-producing glands (Dempsey and Wislocki, 1946; Dempsey 1948). The simplest explanation for the coexistence of this group of reactions within the droplets is that they contain ketoseroids, the only class of substances known to react to the 5 tests enumerated. It is assumed, therefore, that these reactions indicate the presence of the hormones and their precursors. Although any one of the tests may be given by a wide variety of substances, their combined occurrence within the lipid droplets of the ovaries and other steroid-producing glands indicates the value of this group of methods in localizing the sites of steroid-hormone production.

In the sow's ovary, droplets fulfilling the criteria listed above are present in the theca interna of the follicles immediately prior to ovulation and in the theca lutein cells for the first 15 days after ovulation. In addition, droplets which appear in the granulosa lutein cells display the group of tests with the exception that they show no fluorescence. It has been observed before, in other steroid-producing glands, that fluorescence may be relatively difficult to detect (van Dorp and Deane, 1950). It is interesting, as well, that Finkelstein, Hestrin and Koch (1947) have demonstrated that estrogens, apparently by virtue of the high unsaturation of the A ring, are the most fluorescent steroid hormones, whereas those with less unsaturation of this ring (e.g. progesterone, the corticosteroids) are only weakly fluorescent.

The sudanophilic droplets which occur in the basal cells of the granulosa of the follicles in sows' ovaries are occasionally birefringent but show neither a carbonyl reaction nor fluorescence. Similar observations led Dempsey (1948) to postulate that these droplets contain simple triglycerides.

It follows from the present observations that sows' ovaries resemble those of rat (Dempsey and Bassett, 1943) and man (MacKay and Robinson, 1947) in that the steroid hormones are formed in the theca interna of the follicle and in both types of lutein cells. It is impossible, however, to conclude which of these cell types is responsible for the production respectively

of estrogens and progesterone, since the methods are not capable of distinguishing between these several steroids. Ovarian follicles are believed to produce both types of hormone (Hooker and Forbes, 1947), so that a likely site for their production would be the theca interna. The corpus luteum likewise contains both progesterone and estrogens (Doisy, 1939). The histological tests so far developed are incapable of proving the hypothesis, formulated by Dubreuil (1940) and accepted by MacKay and Robinson, that the theca lutein cells produce estrogens and the granuloas lutein cells progesterone.

(2) The group of tests employed appears to have value also in determining the phase of secretory activity. In the adrenal cortex it has been found that the cells are large and their droplets small when secretion is rapid, whereas the cells are small and their droplets large when secretion is not occurring (Deane, Shaw and Greep, 1948). Also, for the adrenal, the birefringent crystals are finest and the carbonyl reactions and fluorescence most intense during active secretion. The attempt has been made to apply those criteria to the ovary in the present study.

In normal follicles during diestrus, the cells of the theca interna are relatively small and their lipid droplets are of moderate size. During the period of the preovulatory swelling, however, these cells hypertrophy; their lipid droplets become smaller but more numerous; the birefringent crystals become finer; and carbonyl reactions and fluorescence emerge. It may be assumed, therefore, that secretion is far more rapid in this period than during diestrus.

Similar signs of activity obtain in the corpora lutea during most of diestrus, from 4 to 14 days after ovulation, particularly in the granulosa lutein cells. Before this period these cells are still relatively small and their droplets quite large. After this period of activity the corpora lutea appear to decline. Concomitant with a shrinking of the cells, the lipid droplets increase in size and the birefringent crystals become coarser. In addition, the droplets become progressively more insoluble in acetone. Such a change in solubility has not been observed in aging corpora of rats' ovaries (Deane and Barker, in press) but resembles that occurring in involuting corpora of monkeys' ovaries (Rossman, 1942) and in the inner part of the adrenal cortex in old or hypophysectomized mice (Jones, 1950). From the more extensive characterization by Jones, it seems possible that these insoluble lipid droplets, which are birefringent, reactive to carbonyl reagents, and fluorescent, may represent a condensation product of steroids. Rossman termed these substances "luteolipin." Similar droplets are found in the theca interna of many atretic and cystic follicles and also in the ovarian interstitial tissue.

The results of chemical analyses of sows' ovaries correlate well with the

histophysiological interpretations made above. For example, Kimura and Cornwell (1938) found small amounts of progestin in corpora lutea for the first 3 days after ovulation, increasing amounts until 15 days, and none thereafter. From 4 to 14 days, the droplets in the corpora are smallest. Bloor, Okey and Corner (1930) found that neutral fats and cholesterol esters exist only in small concentration in corpora lutea during progestational activity. Their percentages rise in degenerating corpora. These rises would appear to account for the increased sudanophilia and birefringence of aging corpora.

Bloor and coworkers also described a high content of phospholipids in active corpora lutea. It is of interest that corpora during the peak of diestrus evinced marked cytoplasmic staining with sudan black and carbonyl reagents. Since this staining could not be prevented by acetone extraction, the cytoplasmic reactions are probably attributable to the presence of phospholipids. Cain and Harrison (1950) have shown for the adrenal cortex that the active, secretory stage is characterized by an abundance of phospholipids demonstrable by Baker's acid hematein test. In general body metabolism, phospholipids have been associated with triglyceride turnover (Sinclair, 1940). However, in the steroid-producing organs, active steroid metabolism may depend similarly on a high concentration of phospholipid.

SUMMARY AND CONCLUSIONS

A group of cytochemical tests (sudanophilia, reactivity with carbonyl reagents, birefringence, fluorescence, and acetone solubility) believed to characterize steroid-secreting glands has been applied to sows' ovaries, examined at all stages of the estrous cycle.

Lipid droplets reacting positively with the entire group of tests occur in the cells of the theca interna of the follicles and in the theca lutein cells. Similar droplets, lacking only in fluorescence, occur in the granulosa lutein cells. For reasons given, it is concluded that all of these cells represent sites of production of ovarian steroid hormones. The methods employed do not permit identification of the individual steroid hormones.

The cells are largest, but their droplets smallest, in the thecal cells of follicles just prior to ovulation, and in the lutein cells during most of diestrus (4 to 14 days after ovulation). Cells of this type are interpreted as being in the active secretory phase.

Lipid droplets occurring in retrogressing corpora lutea, in the theca interna of atretic and cystic follicles and in the interstitial tissue become insoluble in acetone although they continue to respond to all of the other tests employed. These droplets may possibly represent condensation products of steroids.

Acknowledgement

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